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EFFECT OF TEMPERATURE AND MOISTURE ON OVERWINTERING PUPAE OF THE CORN EARWORM IN THE NORTHEASTERN STATES [1]

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INTRODUCTION

A high percentage of the pupae of the corn earworm (*Heliothis armigera* (Hbn.)) that enter hibernation in the Northeastern States during the fall die before the following spring. An effort has been made for a number of years to determine the primary factors responsible for this mortality. In connection with observations under a variety of soil conditions in Virginia, New Jersey, Connecticut, and Massachusetts, the hibernating quarters of the pupae have been studied with particular reference to the effects of moisture, temperature, and physical condition of the soil. Since the conclusions drawn from cage and field observations are in part based on circumstantial evidence, it seemed advisable to study the relation of the soil conditions to pupal survival experimentally in the laboratory and insectary. The work was done at New Haven, Conn., and Arlington, Va., from 1936 to 1938.

HIBERNATING QUARTERS OF THE INSECT

In the Northeastern States the pupae of the corn earworm spend the hibernation period, from about September to June, or at least 9 months, at the bottom of tunnels in the soil. These tunnels are prepared by the larvae and serve as a means of escape for the moths. They range from 1 to about 8 inches in depth, the majority being from 2 to 4 inches, and extend upward to within about half an inch of the surface. The larva packs the wall of the tunnel and lines it with a network of silk. Thus the pupa is located in a dead-air space, which is an excellent insulator, and rests in a chamber lined with a material that is low in thermal conductivity. The cylindrical form of the tunnel, the packing of the walls, and the silken lining keep the tunnel intact over a long period. Alternate freezing and thawing when the soil is moist, invasion by plant roots, and the burrowing of earthworms are some of the important agencies involved in disrupting these tunnels.

EFFECT OF SOIL TEMPERATURE AND MOISTURE

During the long resting period the pupa is subjected to varying conditions of soil temperature and moisture. When rainfall is heavy and water stands on the soil, the tunnels become flooded, and if the soil freezes while the tunnels are flooded, the pupae may become embedded in ice. Precipitation in the form of snow holds the soil moisture at a minimum during the period of low evaporation and also insulates the soil against low temperatures. In soil containing liberal amounts of organic matter the moisture content is higher and disease organisms are probably more prevalent than in mineral soils.

[1] Received for publication August 5, 1939.

The character of the soil itself often governs the conditions to which the pupae are exposed. Thus, sandy soil is usually better drained, and pupae located in it are less often submerged. A tight clay soil with good surface drainage is often favorable for winter survival. The rate of heat conduction in a soil increases as the amount of soil moisture increases, and ice is an even better conductor than water. A pupa attached to frozen soil by a film of ice will therefore give up its heat much more readily than one having a dry surface and resting on dry soil. The high specific heat of water tends to keep the temperature of the moist soil low.

TEMPERATURE

Observations on exposure in dry surroundings showed that a high percentage of pupae would survive a minimum outside air temperature of about 15° F., but exposure at this temperature in moist surroundings resulted in considerable mortality. With a microvoltmeter it was found that the freezing point of pupae maintained in dry air was about 10°.[2] The effect of repeated exposures to subfreezing air temperatures alternated with temperatures above freezing is shown by an experiment summarized in table 1. Ten pupae were exposed to subfreezing air temperatures under dry conditions for 6 periods of 7 to 43 hours and were allowed to warm to 40° in the intervals between exposures. The total time of exposure to freezing temperatures was about 112 hours. It will be noted that these exposures were not fatal.

TABLE 1.—*Survival of (10) corn earworm pupae when repeatedly exposed to freezing air temperatures in an insectary*

Date	Period of exposure	Temperature range	Pupae surviving	Date	Period of exposure	Temperature range	Pupae surviving
	Hours	*° F.*	*Number*		*Hours*	*° F.*	*Number*
Jan. 27–28	24	22–32	10	Feb. 3	8	19–30	10
Jan. 28–30	43	28–33	10	Feb. 4	7	22–31	10
Feb. 2	6	24	10	Feb. 11–12	24	25–31	10

A comparative analysis of 50 hibernating earworm pupae and 100 hibernating European corn borer (*Pyrausta nubilalis* (Hbn.)) larvae on December 11, 1936, showed that the former contained 67.5 percent of moisture and 13.7 percent of fat, whereas the latter contained 55.4 percent of moisture and 20.8 percent of fat. The higher moisture and lower fat content and the exposure to more adverse moisture conditions probably explain why the corn earworm pupae are more susceptible to low temperatures than are corn borer larvae, although in their respective hibernating habitats the earworm pupae are not subjected to such low temperatures as are the corn borer larvae.

MOISTURE

It has long been known that dry soil is favorable for survival of hibernating pupae. When placed in the atmosphere of an ordinary room, or in cardboard or metal boxes, a large proportion of the pupae survive the winter, even though the environment remains warm.

[2] Determined in the laboratory of Roger B. Friend, of the Connecticut Agricultural Experiment Station during the winter of 1937.

The slight loss of their original moisture content under these conditions seems to have little effect on their ability to emerge as moths.

In September 1936, 25 larvae were allowed to dig hibernating quarters in a box containing about 1 cubic foot of dry soil. This box was left indoors. Between July 7 and August 16, 1937, 14 individuals emerged as moths, 9 individuals died as pupae, and 1 died as a larva. In another experiment just a year later 100 larvae were allowed to pupate in 2-ounce tin salve boxes and were kept at room temperature throughout the winter. On April 18, 1938, or 6 months later, 62 pupae were alive.

On November 11, 1937, 25 pupae recovered from outside hibernation cages that had been established about September 15 were placed in an open cardboard box. These pupae were retained in a heated office and were weighed daily for 1 month. On November 12 the average weight was 0.541 gm., and by December 11 it had decreased to 0.486 gm. Thus, in a single month of exposure in a heated room these pupae lost 10 percent of their weight. Some weight was lost each day, but the rate of desiccation was highest during the first 5 days of the experiment. Twenty-four individuals emerged as moths between December 13 and April 18. The ability of the pupae to withstand loss of moisture allows the insect to survive dry conditions during both hibernation and the growing season.

The pupa is covered with an oily material, which permits it to shed water. When the humidity of the burrow is high, small beads of moisture appear on the surface of the pupa, especially when the surrounding soil is warm. Normal soil moisture (without flooding) does not seem to be injurious, but probably is advantageous in maintaining a moisture balance.

In contrast to a loss of 10 percent of their weight in 1 month by pupae kept under dry conditions in a heated room, another lot of 25 pupae kept between moist paper towels in a cool room lost only 1.7 percent of their weight in over 2 months. On November 25 these pupae weighed on an average 0.529 gm. and on January 29, 0.520 gm. On May 10, 22 of these pupae were alive. This was a higher rate of survival than that obtained in any other lot of pupae that the writers have carried through hibernation.

In their burrows the pupae usually encounter a moist environment, but they are subjected to periods of drought and to periods of submergence during or following heavy rainfall. During such dry or wet periods they lose or gain weight. Changes in weight of pupae subjected to different successive degrees of moisture are shown in the experiment summarized in table 2. When exposed to dry air (simulating drought conditions) loss of weight was rapid; when in contact with moist earth (simulating the usual pupal environment) a slight loss of weight occurred; when submerged in artificial burrows (simulating the conditions during heavy rainfall) pupae gained slightly in weight. Under the first two conditions all the pupae survived, but the last condition was hazardous and caused a heavy mortality.

When hibernation cages are examined in the spring, pupae are often found which appear normal in all respects except that they are immobile. When exposed to room temperatures, some of these individuals are revived and emerge as apparently normal moths. In the

experiments that have been described similar individuals were encountered. The observations indicate that the causes of death in the hibernation cages and in the experiments are similar and that considerable pupal mortality is caused by the smothering effect of water, some of which may be absorbed by the tracheal system.

TABLE 2.—*Average changes in weight of 2 lots of 15 corn earworm pupae subjected to different successive conditions of moisture*

Lot	Environment	Period of exposure	Average weight of 1 pupa		Gain (+) or loss (−) in weight	Pupae surviving
			At beginning	At end		
			Gram	*Gram*	*Percent*	*Number*
1	Dry air of room	Dec. 9–14	0.550	0.521	−5.3	15
	Flooded burrows at room temperature	Dec. 14–20	.521	.526	+1.0	7
2	Dry air of room	Dec. 9–14	.526	.484	−8.0	15
	Buried in moist earth at room temperature	Dec. 14–20	.484	.482	−.4	15
	Dry air of room	Dec. 20–27	.482	.466	−3.3	15
	Submerged in burrows at room temperature	Dec. 27–Jan. 3	.466	.472	+1.3	1

EFFECT OF SUBMERGENCE IN WATER

An observation in soil saturated with water at Charlottesville, Va., in August 1932, revealed the pupae floating in the top of the tunnels. Apparently such pupae recede to the bottom of the tunnels with the water level or work back to their normal position by their wriggling movements. Experiments in the laboratory with pupae placed in glass tubes (of a diameter similar to that of the hibernating burrows) partly filled with distilled water gave information on the proportion of pupae that would float, how long they would float, and the effect of floating on survival. The results of such an experiment are summarized in table 3. At the end of the period of treatment 62.5 percent of the pupae were floating. There was variation in the position of some of the pupae from day to day. Thus, 57.5 percent floated during the entire period of observations, 20 percent were submerged for the entire period, 17.5 percent of those that floated at first sank later, and 5.0 percent changed their status more than once, that is, they floated at first, then sank, and later floated again. These data show that, if pupae floated, some survived for as long as 20 days, whereas if they were submerged without floating they died within less than 10 days.

TABLE 3.—*Effect of submergence of corn earworm pupae for different periods in artificial burrows partly filled with water* [1]

Duration of immersion (days)	Pupae floating at end of period	Pupae submerged at end of period	Survival of pupae that floated	Survival of pupae that sank
	Number	*Number*	*Number*	*Number*
5	4	6	2	4
10	7	3	5	0
15	5	5	2	0
20	9	1	4	0
Total	25	15	----	----

[1] These experiments were conducted in an unheated basement room, the air temperature of which was about 60° F. 10 pupae were immersed for each period.

Of a series of 25 pupae submerged at 75° F. in lots of 5 for intervals of from 2 to 10 days, 20 percent survived. In a similar series submerged for like periods at 40°, 84 percent survived. The much higher mortality in water at 75° was due, no doubt, to the higher rate of metabolism with insufficient oxygen. This is probably an important factor in mortality of hibernating pupae under high soil-moisture conditions in the spring when higher soil temperatures prevail.

The importance of high soil moisture in the summer mortality of the soil stages of the earworm is indicated in the results of an experiment at Arlington, Va., begun August 20, 1937. A total of 312 ears infested with larvae, mostly in the fifth and sixth instars, were impaled in cages. Most of the larvae entered the soil during the following 10 days, which was a period of heavy rainfall. On September 15 these cages were examined, with the following results: Living pupae, 122; dead pupae, 129; pupal exuviae, 24; total mortality, 46.9 percent.

General observations in hibernation cages confirm these results. It seems, therefore, that pupae may survive flooding for short periods following heavy rainfall, and that the rate of mortality increases as the temperature increases. The ability of the pupae to float in their burrows doubtless accounts for the high percentage of survival that is often encountered after rather prolonged wet periods.

COMPARATIVE WINTER MORTALITY OF PUPAE RESTING ON A DRY AND ON A WET MEDIUM

During the winters of 1936–37 and 1937–38 two lots of pupae, one lot resting on a dry medium and the other on a moist medium, were exposed to outdoor temperatures at Arlington. In 1936–37 another lot was exposed on moist sand. These pupae had been collected from soil in hibernation cages or field plots about the middle of November and were placed in 2-ounce salve boxes containing about three-eighths of an inch of sand or loam. The pupae used in 1936–37 matured on sweet corn (*Zea mays* var. *rugosa*). In 1937–38 some of the pupae had been collected at Moorestown, N. J., and at Marietta, Ohio, on Evergreen sweet corn; others had been collected at Arlington, the larvae having matured on field corn (*Z. mays*) in the dough stage. No moisture was added to the boxes containing the dry sand or soil, but moisture was added occasionally during the winter to the boxes started with moist sand or soil. Two holes of uniform size were punched in the lid of each box to provide ventilation.

The survival of these pupae throughout the winter is given in tables 4 and 5. The relative survival on moist media and also on dry media was similar in both years; however, the actual survival on the dry media was nearly twice as high at the end of the second-year period as at the end of the first-year period. A great reduction in surviving pupae occurred during the early part of the winter of 1936–37, when there were rapid drops to below-freezing temperatures. It is believed that these sudden exposures account for most of the difference in survival between the 2 years.

The effect of differences in the thermal conductivity of dry and moist environments on the pupal mortality was strikingly brought out in the 1936–37 experiments on moist and dry soil. The pupae

had been collected in the field on November 23 and stored at about 70° F. until November 30 in order to eliminate as much as possible insects injured in handling. The temperature at the beginning of the exposure was 40°. It dropped to below freezing after a few hours, reaching a minimum of 15° for about 2 hours early the next day. On December 1 the temperature was above freezing for about 5 hours, reaching a maximum of 42°, and dropped to a minimum of 29° early on December 2. An examination on December 2 showed a mortality of 56.9 percent on moist soil and 15.7 percent on dry soil. Since the internal moisture of the pupae in this instance could hardly have changed enough to affect their freezing point, it is evident that the pupae resting on moist soil gave up their heat more readily than those on dry soil.

TABLE 4.—*Survival of corn earworm pupae when resting on moist sand and on moist and dry soil in an insectary at Arlington, Va., throughout the winter of 1936–37*

Observation period [1] beginning—	Minimum temperature	Days below freezing	Survival of pupae at end of period			Observation period [1] beginning—	Minimum temperature	Days below freezing	Survival of pupae at end of period		
			100 pupae on moist sand	51 pupae on moist soil	51 pupae on dry soil				100 pupae on moist sand	51 pupae on moist soil	51 pupae on dry soil
	° F.		*Percent*	*Percent*	*Percent*		° F.		*Percent*	*Percent*	*Percent*
Nov. 24	19	3	30.0	.....	.....	Jan. 23	30	2	.0	3.9	50.9
Nov. 30	15	2	13.0	43.1	84.3	Jan. 30	21.5	5	.0	.0	50.9
Dec. 2	27	4	13.0	39.2	76.5	Feb. 6	20	5	.0	.0	49.0
Dec. 7	22.5	2	9.0	7.9	74.5	Feb. 13	22	5	.0	.0	49.0
Dec. 11	27	4	8.0	7.9	74.5	Feb. 20	19	6	.0	.0	33.3
Dec. 16	21	6	6.0	5.9	58.8	Feb. 27	19	3	.0	.0	27.4
Dec. 24	27	3	4.0	3.9	54.9	Mar. 6	22	5	.0	.0	27.4
Jan. 4	25	1	4.0	3.9	52.9	Mar. 13	28	6	.0	.0	27.4
Jan. 9	35	0	3.0	3.9	50.9	Mar. 20	26	2	.0	.0	27.4
Jan. 16	33.5	0	3.0	3.9	50.9	Mar. 27	31	2	.0	.0	27.4

[1] Each observation period begins at the conclusion of the next preceding period.

TABLE 5.—*Weekly survival of corn earworm pupae when resting on moist and dry sand in an insectary at Arlington, Va., throughout the winter of 1937–38*

Week beginning—	Temperature		Days below freezing	Survival of pupae at end of period		Week beginning—	Temperature		Days below freezing	Survival of pupae at end of period	
	Mean minimum	Minimum		152 pupae on moist sand	148 pupae on dry sand		Mean minimum	Minimum		152 pupae on moist sand	148 pupae on dry sand
	° F.	° F.		*Percent*	*Percent*		° F.	° F.		*Percent*	*Percent*
Nov. 13	40.6	33	0	100.0	100.0	Jan. 22	27.0	15	5	5.9	56.8
Nov. 20	24.6	20	5	71.7	96.7	Jan. 29	26.4	15	3	3.3	54.1
Nov. 27	35.4	26	2	71.1	96.7	Feb. 5	31.9	28	3	3.3	54.1
Dec. 4	23.0	16	5	52.0	90.5	Feb. 12	32.6	23	3	3.3	52.7
Dec. 11	22.4	14	5	18.4	81.8	Feb. 19	32.4	27	3	3.3	52.7
Dec. 18	34.0	27	2	17.8	78.4	Feb. 26	25.4	19	5	1.3	52.0
Dec. 25	30.4	22	4	17.8	77.7	Mar. 5	30.3	21	3	1.3	52.0
Jan. 1	30.0	24	5	17.8	76.4	Mar. 12	39.7	32	1	1.3	52.0
Jan. 8	26.1	18	6	13.8	75.0	Mar. 19	45.3	34	0	1.3	52.0
Jan. 15	23.6	14	6	8.6	66.9	Mar. 26	42.3	31	1	1.3	52.0

The rate of pupal survival in relation to temperature and moisture for the winter of 1937–38 is also shown graphically in figure 1. The heaviest mortality occurred before December 18 among the individuals on moist sand. The minimum temperature recorded during this period was 14° F. Temperatures of 15° or below were recorded on 4 days during December and January. December had the lowest mean temperature of the winter months. Evidently some individuals were more susceptible to freezing temperatures than others, particularly among the group on moist sand. After December 18 the remaining pupae on wet sand suffered less mortality than those on dry sand. That pupae on dry sand were injured by low temperatures was evident from a mottled brown to dark-brown appearance of

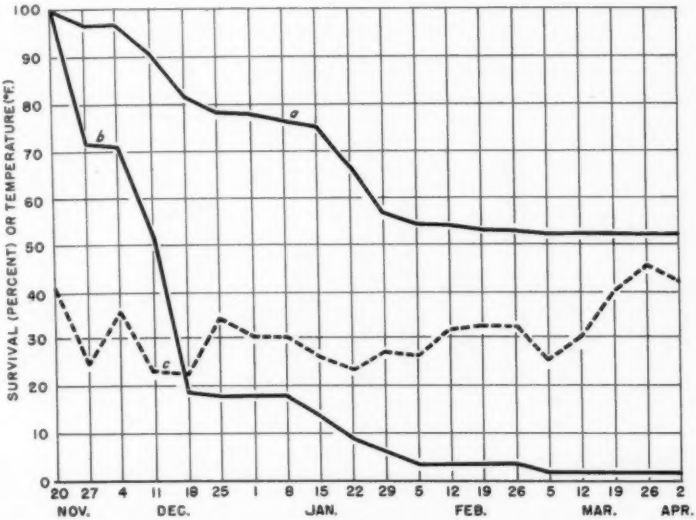

FIGURE 1.—Weekly survival of hibernating pupae in salve boxes on dry and on moist sand, exposed to outside temperatures during the winter of 1937–38, Arlington, Va.: *a*, Survival on dry sand; *b*, survival on moist sand; *c*, mean minimum temperature to which the pupae were exposed.

some individuals. Such pupae usually died within a few weeks after receiving this injury.

Of the 14 pupae that survived the winter of 1936–37, 12 produced moths and 2 died between May 31 and July 13. In 1938, 69 moths emerged and 10 pupae died between May 24 and July 8 from a total of 79 pupae that were surviving on April 2. In general, the data are significant in showing that hibernating pupae of the earworm are able to withstand rather severe soil temperatures, particularly when the soil is dry.

The only pupae that survived on moist sand or soil during the two winters developed from larvae that matured on field corn in the dough stage. There was very little difference in mortality of pupae resting on a dry medium whether sweet or field corn had been the host, nor

was there any significant difference among the lots from the different collection points.

The results are similar to those observed in hibernation experiments for a number of years, particularly in the winter of 1935–36, when an unusually high mortality followed heavy rains in January and a rapid drop in temperature which continued at a low point for approximately 1 month. During the early part of this period there was little protection from snow cover, and frost penetrated the wet soil to a depth of about 2½ feet. This condition was followed by snowfall, which acted as a good insulator for holding the soil temperature at or below the freezing point. Under these conditions the temperature in clay containing some fine sand and much organic matter reached a minimum of 22.5° F. at a depth of 4 inches.

EFFECT OF AVAILABILITY OF AIR

Although the vital processes in animals are at a low ebb during hibernation, a certain amount of respiration is necessary. During the

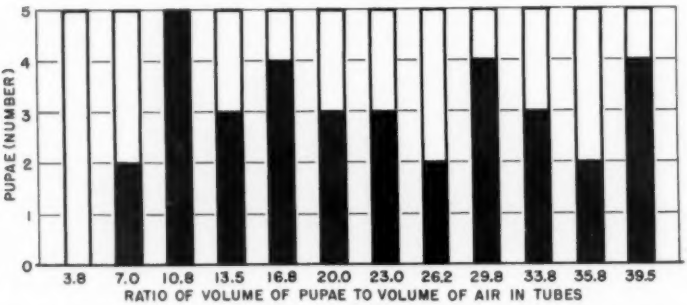

FIGURE 2.—Survival of pupae after 6 months at 40° F. in tubes in which the volume of available air ranged from 3.8 to 39.5 times the volume of the pupae. Each column represents five pupae in individual tubes having the same volume of air, the solid portions showing number of pupae alive at the end of the experiments.

winter, when the ground is frozen for several weeks at a time, the pupae of the corn earworm are often sealed in their burrows with ice. The question arises, Do the pupae smother under such conditions?

A total of 60 pupae were placed individually in glass tubes of a diameter similar to that of the insects' hibernating tunnels (⅜ inch) and of 12 different lengths ranging from 1½ to 12½ inches. The ends of each tube were closed by cork stoppers cut flush and then sealed with waterproof glue. The volume of air in these tubes ranged from 1.9 to 16.2 cc. It was found that the average displacement of air by a pupa was about 0.4 cc. The volume of air in the tubes therefore ranged from 3.8 to 39.5 times the volume of the pupa. The tubes were stored in a cold room at about 40° F. from October 9, 1936, until May 5, 1937, a period of 208 days. At the end of the period 35 pupae, or 58 percent, were found to be alive (fig. 2). Except for the individuals confined in the smallest volume of air, mortality was probably due to causes other than lack of air.

Of 120 pupae in individual unsealed salve boxes under similar temperature conditions and for a similar period, 44 percent were alive on April 7, 1937.

These experiments indicate that, in the tunnels of average length, it is unlikely that pupae die from lack of air during hibernation, even when the soil is frozen solidly about the burrows for prolonged periods.

EFFECT OF BEING EMBEDDED IN ICE

Following heavy rainfall and a sudden drop to subfreezing temperatures pupae may become partly or wholly embedded in ice. This condition was simulated in a refrigerator. Ten pupae were taken from storage at 40° F. and placed at the bottom of small paper cups filled with water. The cups were then placed in a tray in the refrigerator away from contact with the freezing unit. The air temperature at the location of the pupae was about 26° F., and an exposure of at least 8 hours was necessary to freeze the water solidly. After 7 hours the water had frozen except at the center of the cups. All the pupae survived this treatment. When exposed for 18 hours 1 of 10 pupae survived, and when exposed for 24 hours all the pupae died. In the last 2 instances the pupae had been embedded solidly in ice for approximately 10 and 16 hours, respectively.

In January, 10 pupae in similar cups of water were exposed for 24 hours outdoors at temperatures ranging from 22° to 32° F. At the end of this period they were embedded solidly in ice. Only 1 pupa survived this treatment. On February 2, 10 pupae were exposed under similar conditions for 6 hours at 24°, and although they were embedded solidly in ice at the end of the period, 8 survived. Another series of 10 pupae were exposed similarly on February 4–5 for 24 hours at a temperature of 30°–32° most of the time. At the end of the period the water in the center of the cups had not frozen. All these pupae survived.

EFFECT OF EARTHWORM ACTIVITY AND OF BEING EMBEDDED IN SOIL

In localities where the soil does not freeze repeatedly and when they are not disrupted by the soil fauna or by plant roots, the pupal tunnels, because of their firm construction, are often found to be in perfect condition as much as a year after being formed. In the Northeastern States several factors cause disruption and filling in of the tunnels. Important among these factors are heaving and settling of the soil due to alternate freezing and thawing. Earthworms disrupt a high percentage of the tunnels in soils favorable to them, especially during wet periods when their activity is concentrated near the surface.

The probable effect on emergence of moths from tunnels that have been partly or wholly filled with the droppings of earthworms has been pointed out by Phillips and Barber.[3] The present writers' observations on hibernation cages located in clay-sand soil high in organic matter at Arlington from 1934 to 1937 indicated that a considerable mortality among hibernating pupae was caused directly or indirectly by intense earthworm activity. In 6 cages, each stocked

[3] PHILLIPS, W. J., and BARBER, GEORGE W. A STUDY OF HIBERNATION OF THE CORN EARWORM IN VIRGINIA. Va. Agr. Expt. Sta. Tech. Bul. 40, 24 pp., illus. 1929.

in the fall with 100 full-grown larvae, there was no survival in the 3 years of the experiment. By the middle of April the soil was perforated with earthworm tunnels and the tunnels of the earworm had been practically obliterated. The pupae had collapsed and disintegrated to such an extent that only the exoskeletons remained. This rapid disintegration of pupae embedded in the droppings was probably brought about by the nitrogenous environment. In 6 similar cages containing a sandy loam in which earthworms were much less active the pupal survival in the 3-year period was 9.8 percent.

Since soils favorable for earthworms are rich in organic matter and have a high moisture-holding capacity, which in itself has been shown to be unfavorable for survival, it is difficult to determine under field conditions to what extent the droppings of earthworms are directly toxic to the pupae.

The mild winter of 1936–37 at Arlington practically eliminated freezing soil temperatures as a factor in winter mortality. The soil moisture was high, and earthworm activity was concentrated near the surface for a considerable period. In table 6 are given the results of examinations in a field plot in which there was a heavy population of earthworms. There was little, if any, pupal mortality on November 23 that could be attributed to earthworm activity in the tunnels. By March 22 the survival had dropped to 38.9 percent, and the mortality continued at about the same rate from March 22 to April 19.

TABLE 6.—*Survival of hibernating pupae of the corn earworm in a field plot having a high earthworm population, Arlington, Va., 1936–37*

Date	Pupae recovered	Living pupae		Dead pupae		Survival
		Free or partly embedded	Embedded	Free or partly embedded	Embedded	
	Number	*Number*	*Number*	*Number*	*Number*	*Percent*
Nov. 23	184	170	0	14	0	92.4
Mar. 22	95	37	0	25	33	38.9
Apr. 19	101	24	0	14	63	23.8

The heavy rainfall during the winter months undoubtedly accounted for most of the mortality, since of the 58 dead pupae recovered on March 22, 25 were not embedded. A decrease in the number of dead pupae not embedded on April 19 indicates that many of the embedded specimens had died prior to becoming embedded. All the living pupae were found in tunnels, in which they were permitted some degree of movement. Movement of the abdomen appears to be essential for the insect to survive embedding in moist soil for any length of time. In the spring 8 living pupae were found with the thoracic region rather firmly embedded but free to move the abdominal segments.

In experimental shallow embedding on May 8, 1937, 10 pupae were covered with mud and allowed to dry on a paper tray. Owing to the oily nature of their integument, they freed themselves of much of the mud by wriggling. From these pupae 8 moths emerged, but in 2 instances the mud adhered too closely for the moths to escape from the pupal cases. From 10 pupae covered with a layer of mud three-fourths of an inch thick, which later dried and hardened, 1 pupa

was alive on July 1 and 9 had become moths but were unable to free themselves from the pupal cases. Of 10 pupae covered with a ¾-inch layer of moist packed soil, all individuals wriggled to the surface and emerged as moths. There was complete emergence from 10 pupae placed on a paper tray out of contact with soil.

The data presented substantiate the previous indications that soil moisture is an important factor contributing to the mortality of hibernating pupae. There is, however, no conclusive evidence that the biotic and chemical conditions brought about and associated with intense activity of earthworms in the soil at hibernating levels are directly toxic to the pupae. Earthworm activity at and above the hibernating level of the pupa coincides with high soil moisture, and obstructions in the tunnels under such conditions subject pupae to longer than normal submergence.

EFFECT OF THERMAL CONDUCTIVITY IN SOILS

It has been shown that pupae succumb in much greater numbers when exposed to the same temperature while resting on a moist medium than on a dry medium. When the conditions in soils varying in moisture content are considered, it is very evident that the transmission of heat from the pupae is an important factor in mortality. As water is transformed from the liquid to the solid state, the thermal conductivity is more than quadrupled. The thermal conductivities of various substances involved, expressed in calories, are as follows: Air 0.000056, water 0.0012, ice 0.005, compact snow 0.00051, dry sand 0.00093, dry soil 0.00033, and silk (similar to chitin in nature) 0.000095[4] When the insulating efficiency of dry air, dry soil, and a dry exoskeleton is considered in addition to the reduced water content of pupae hibernating under dry conditions, the primary reason for a higher survival in such an environment is quite obvious. Pupae resting in wet soil, a good conductor of heat, have a moist exoskeleton and are practically fused to the soil when it freezes, thus allowing heat to flow readily from their bodies.

Experiments on hibernation in this area in a variety of soils have shown that the rate of survival is highest in well-drained soil, usually sand. The reason for this may be inferred from a study by Bouyoucos[5] of soil temperatures under laboratory and field conditions. He found that the specific heat of gravel, sand, loam, clay, and peat soils did not differ materially, but that under field conditions their water-holding capacity differed greatly, and that, because of the high specific heat of water, the differences in water content accounted for the differences in soil temperature. The temperatures of these soils varied but little during the winter, the gravel and the sand being slightly colder. The lower water capacity of the gravel and sand, the greater radiation of heat in these types of soil, and the higher thermal conductivity resulted in their thawing out earlier and being more responsive to air temperatures in the spring. Organic matter increased the water-holding capacity of the soil, peat holding about

[4] These figures were taken from a compilation in the following: CHEMICAL RUBBER COMPANY. HANDBOOK OF CHEMISTRY AND PHYSICS, A READY-REFERENCE BOOK OF CHEMICAL AND PHYSICAL DATA. Ed. 20, 1951 pp., illus. Cleveland, Ohio. 1935. See pp. 1301–1302.

[5] BOUYOUCOS, GEORGE J. AN INVESTIGATION OF SOIL TEMPERATURE AND SOME OF THE MOST IMPORTANT FACTORS INFLUENCING IT. Mich. Agr. Expt. Sta. Tech. Bul. 17, 196 pp., illus. 1913.

25 times as much water as the mineral soils sand and gravel. The factors thus shown to be characteristic of gravelly and sandy soils—i. e., more rapid warming up in the spring and lower water content, together with the good drainage which is usually characteristic of mineral soils like sand and gravel—are no doubt responsible for the more successful hibernation found in such soils.

It was shown in table 2 that in room atmosphere the pupae lose moisture rather rapidly, but that moisture is again absorbed upon subsequent exposure. The moisture is perhaps absorbed by the sclerotic exoskeleton and the tracheal system. The freezing of this absorbed moisture is apparently injurious to the tissues affected. This type of injury was particularly apparent in the integument of pupae resting on dry sand. When such pupae were exposed to temperatures below 20° F., some individuals had a mottled appearance, the dark areas discernible in the integument resembling typical frostbite. Pupae so injured usually died within a few weeks. Pupae killed by freezing while resting on moist sand were somewhat distended and rather uniformly dark brown, eventually turning almost black.

SUMMARY

In order better to interpret observations in the field, laboratory and insectary experiments have been conducted at New Haven, Conn., and Arlington, Va., on the effect of moisture and temperature on hibernating pupae of the corn earworm (*Heliothis armigera* (Hbn.)).

The pupa hibernates in the soil from about September to June at the base of an upward-sloping cylindrical tunnel, usually at a depth of from 2 to 4 inches. The wall of the tunnel is packed and lined with silk, which helps to hold it intact for long periods of time. Heaving of the soil caused by alternate freezing and thawing and invasion of plant roots and earthworms are important agencies in disrupting the tunnels during the hibernating period, causing pupae to become embedded in the soil and die, and also interfering with exuviation and emergence. Well-drained soils, particularly sandy ones, provide conditions favorable for survival.

The freezing point of pupae maintained in dry air was found by the microvoltmeter method to be about 10° F. Analyses of some hibernating pupae, made in December, showed 13.7 percent of fat and 67.5 percent of water as compared with 20.8 percent of fat and 55.4 percent of water in hibernating European corn borer larvae.

Hibernation indoors in the atmosphere or in salve boxes in a heated room results in but little mortality and has little effect on the ability of moths to emerge. Pupae exposed 1 month in a heated room lost 10 percent in weight in contrast with 1.7 percent for pupae kept between moist paper towels in a cool room. When pupae are submerged in water after losing weight in dry air they gain slightly in weight. This gain seems to be due to absorption of water by the exoskeleton, the tracheal system, or both.

The pupae are able to float in flooded tunnels. In the laboratory they were observed to float, sink, and then float again. Individuals that did not float died within a short time. The ability to float in flooded tunnels permits survival in periods of heavy rainfall. The mortality among submerged pupae increases with a rise of tempera-

ture. At 75° F. 20 percent survived submergence for 10 days, in contrast to 84 percent at 40°.

During the winter of 1936–37, 27.4 percent of the hibernating pupae exposed in an outdoor insectary at Arlington, Va., survived in 2-ounce salve boxes on dry soil, whereas there was no survival on moist soil. In the winter of 1937–38, under similar conditions, 52 percent survived on dry sand and 1.3 percent on moist sand. The minimum temperature during these two winters was 14° F. The higher rate of survival on dry soil is attributed to the better insulating efficiency of a dry environment and of a dry pupal exoskeleton and to the lower water content of pupae hibernating under dry conditions. Pupae resting on wet soil are more susceptible to below-freezing temperatures because of a higher thermal conductivity of moist soil, more body water, and a moist exoskeleton, which practically becomes fused to the soil when it freezes, allowing the body heat to be given up readily.

In sealed tubes ranging from 1½ to 12½ inches in length there was survival in all but the shortest at the end of 208 days of exposure at 40° F. Of 10 individuals, 1 survived being frozen in ice for 18 hours. An exposure of 24 hours was fatal.

THE *D Rs P* LINKAGE GROUP IN SORGHUM[1]

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INTRODUCTION

This paper reports a linkage group of three factors in sorghum (*Sorghum vulgare* Pers.) and the independence of this group from several other factors that have been reported previously by the writers or by other workers. The linked factors are those for dry and juicy stalks (*Dd*), red and green seedling stems (*Rsrs*), and purplish and brownish plant color (*Pp*). These linkages were studied in the coupling phase.

REVIEW OF LITERATURE

Rangaswami Ayyangar (*6*)[2] reported that Benson and Subba Rao in 1906 recorded the observation that dry-stalked sorghums have a white midrib and that juicy-stalked varieties have a dull midrib. In 1916 Hilson (*2*) reported a single-factor difference between dry and juicy stalks, with dry stalks dominant to juicy. Swanson and Parker (*14*) assigned the symbols *D* and *d* to the factors for dry and juicy stalks, respectively, and illustrated the differences in midrib color. These authors found a higher percentage of smutted plants in rows homozygous for juicy stalks than in rows with dry-stalked plants. It was suggested, however, that the association may have been between smut susceptibility and sweetness rather than smut susceptibility and juiciness. Rangaswami Ayyangar et al. (*9*) reported that juiciness and sweetness are inherited independently. Rangaswami Ayyangar and Kunhi Koran Nambiar (*7*) found independent segregation of factors for juiciness and a character called mechanical tissue brown.

In 1930 Reed (*11*) described and illustrated the contrasting characters red and green seedling stems. He found seedling stem color to be monogenic in inheritance, with red stems dominant to green. Karper and Conner (*3*) reported linkage between seedling stem color and a seedling albino, with 41.34 percent crossing over. A two-factor segregation for seedling stem color was reported by Woodworth (*15*) in a cross between shallu and Black Spanish broomcorn.

Rangaswami Ayyangar et al. described and illustrated in color (*10, pl. XLII*) the contrasting plant colors purple and brown. Purple was dominant to brown, and a 3:1 segregation was obtained in F_2. The symbols *P* and *p*, respectively, were assigned to the factor pair for these plant colors. Rangaswami Ayyangar et al. (*8*) reported a

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[2] Italic numbers in parentheses refer to Literature Cited, p. 730.

linkage between the factor pair determining plant color and that for juiciness of stalk, with 30 percent crossing over in the F_2 repulsion phase.

With the exception of the male sterile character, the literature for all characters mentioned in this paper has been reviewed recently in connection with a report on the *Q B Gs* linkage group (*13*).

A male sterile character, recessive to the normal condition and characterized by incompletely developed anthers, was described in 1937 (*12*). This factor pair was given the symbols *Ms* and *ms*.

DESCRIPTION OF CHARACTERS

JUICINESS

In some varieties of sorghum the leaf midribs have a chalky-white appearance. If a mature stalk of one of these varieties is split the pith is found to be relatively dry. In other varieties midribs have a dull or opaque appearance, and the stalks of these varieties contain appreciable amounts of juice. That the dull appearance of the midrub in juicy varieties is due to juice may be shown by breaking a leaf and pressing juice out of the midrib, after which it has much the same chalky appearance as midribs of dry varieties. Little or no juice can be pressed from the midribs of dry varieties. The contrasts in midrib color are well shown in illustrations by Swanson and Parker (*14*) and by Rangaswami Ayyangar et al. (*8*).

Seedlings and new leaves of all sorghums have dull midribs. The white midrib of dry varieties starts as a narrow streak in the center and gradually spreads toward the margins until the whole midrib is white. Juicy varieties usually have a white streak through the center of each midrib before the plants are mature. However, the white streak begins to show much later in the plant's development than in the case of dry varieties, and the margins of the midribs are always dull. Midrib appearance (color) is generally used to separate dry and juicy plants in segregating populations, and usually the separations can be made readily.

SEEDLING STEM COLOR

Many varieties of sorghum, including broomcorn, Sudan grass, most of the milos and kaoliangs, and many of the sorgos, have red seedling stems. Other varieties, including the Blackhull kafirs and most of the Orange group of sorgos, have green seedling stems. Frequently the contrast between red and green can be observed as soon as the coleoptiles appear at the surface of the ground, but the red color becomes more intense after exposure to light for a few days. In red seedlings the color is most intense in the coleoptile, but sometimes the sheath and under side of the blade of the first to third leaves also are colored red wholly or in part. Below the surface of the soil or in the absence of light, both red and green seedlings are usually white. The contrast between red and green seedling stems was clearly illustrated by Reed (*11, figs. 24 and 25*). Color varies somewhat in intensity and shade between varieties; in Sumac sorgo, for instance, the color is dark purplish red, whereas in milo it is salmon red. Within the experience of the writers, however, the presence or absence of red seedling stem color is due to a single-factor pair, and such variations in intensity or shade as exist between varieties are

probably caused by unidentified modifiers. The F_2 generation of the shallu × Black Spanish broomcorn cross, wherein Woodworth (*15*) reported a two-factor segregation, has been grown at Chillicothe, Tex., only once, and environmental conditions were such that phenotypes could not be separated satisfactorily.

Under field conditions, environment influences expression of seedling stem color to such an extent that it is often impossible to separate classes accurately in a segregating population. The exact environmental factors that are responsible for failure of full development of color have not been determined, but trouble in separating phenotypes usually occurs if seedlings emerge when soil is relatively dry and temperature high, particularly if wind is high also. Under such conditions, the red color of red seedlings fails to develop its usual intensity and the green color of green seedlings becomes dark or somewhat gray, and the two types are hard to separate. It is then necessary to grow seedlings of the next generation to determine seedling stem color.

PLANT COLOR

Rangaswami Ayyangar et al. (*10*) have reported that nearly all sorghums except one group—represented in the United States by the variety shallu—have purple pigmentation. The purple pigmentation is conspicuous in leaf sheaths and glumes. In the absence of purple pigmentation, tissues are brownish. Purple is a simple character dominant to brown, and the symbols *P* and *p*, respectively, are used for the factor pair concerned. Purple-pigmented plants are divided into two classes, reddish purple with red glumes and blackish purple with black glumes, represented by the factor pair *Qq*. Phenotypes are illustrated in color (*10, pl. XLII*).

So far as known to the writers, shallu and Leoti sorgo are the only commercial varieties grown in the United States that have the brown plant color. Shallu is an introduction, and the parentage of Leoti is unknown. It might have been derived from a shallu hybrid. In segregating populations, reddish-purple or blackish-purple plants are easily distinguished from brown plants after they have reached a stage where foliage diseases or injuries cause the development of spots on the leaf sheaths and blades. The characters can be distinguished in seedlings a few days after mechanical injury or by examination of injured or decaying roots. Seemingly there is no distinguishable effect of the factor pair *Qq* on leaf sheaths of brown (*pp*) plants, but an effect on glume color is readily apparent. On brown plants *Q* produces sienna-colored glumes instead of the red found in purple plants, whereas the allelomorph, *q* gives mahogany-colored glumes instead of black. In populations heterozygous for both of these plant colors, *Pp* and *Qq*, a 9 : 3 : 3 : 1 segregation is obtained.

LINKAGE OF D, Rs, AND P

Linkage between the genes for juiciness (*Dd*) and seedling stem color (*Rsrs*) was observed by the writers at Chillicothe in 1930, and this observation was reported by Martin (*4*) in a summary of sorghum linkages published in 1936. Genes for juiciness and plant color (*Pp*) were found to be linked in 1935, and this was mentioned briefly in the 1936 Texas station annual report (*5*). Rangaswami Ayyangar

et al. (*8*) in 1937 reported experiments showing linkage between juiciness and purple plant color, with 30 percent crossing over.

No triple recessive (*drsp*) plant was located until the late summer of 1936, when one was found in the progeny of a Leoti × Ajax cross. Crosses with two triple-dominant varieties, Brown kaoliang, S. P. I.[3] 38085, and Chusan Brown kaoliang, S. P. I. 23231, were made the same year, but only a few seeds were obtained. One F_1 plant of each cross grown in a greenhouse at Washington, D. C., during the winter of 1936–37, supplied small F_2 populations at Chillicothe in 1937. Additional F_2 and F_3 populations were grown in 1938. In the latter season classification of seedling stem color was somewhat questionable and, instead of labeling the seedlings, one head of each plant was bagged and after maturity seed was planted at the base of the plant to determine seedling stem color factors of the parent. The phenotypes for juiciness and plant color were separated readily.

The single-factor inheritance of each character in these populations is shown in table 1. The parental combinations and recombinations of the three characters are given in table 2. The indicated order of genes, with cross-over percentages, is *D* (10.9) *Rs* (16.4) *P*. The total cross-over percentage (27.3) agrees satisfactorily with the 30 percent reported by Rangaswami Ayyangar et al. (*8*).

TABLE 1.—*Total classes of juiciness (Dd), seedling stem color (Rsrs), and plant color (Pp) in F_2 and F_3 segregating populations of (Leoti × Ajax—drsp line) × Brown kaoliang grown in 1937 and 1938, showing monogenic segregation for each of the characters*

Factors	Total population	Classes			χ^2
		Dominant	Recessive		
	Number	*Number*	*Number*	*Percent*	
Juiciness, *Dd*	2,091	1,576	515	24.63	[1] 0.163
Seedling stem color, *Rsrs*	2,091	1,588	503	24.06	1.020
Plant color, *Pp*	2,091	1,576	515	24.63	.163

[1] $P>0.05$ for segregation of each factor pair.

TABLE 2.—*Parental combinations and recombinations of juiciness (Dd), seedling stem color (Rsrs), and plant color (Pp) in F_2 and F_3 populations coupling phase of (Leoti × Ajax—drsp line) × Brown kaoliang grown in 1937 and 1938*

Combinations and recombinations	Factors	Population		Percent
		Number	Total	
Parental combinations	*DRsP*	1,303	1,562	74.701
	drsp	259		
Recombinations between *Dd* and *Rsrs*	*dRsP*	108	187	8.943
	Drsp	79		
Recombinations between *Rsrs* and *Pp*	*DRsp*	165	301	14.395
	drsP	136		
Double recombinations	*DrsP*	29	41	1.961
	dRsp	12		
Total			2,091	
Recombinations and double recombinations	*Dd–Rsrs*			8.943+1.961=10.904
	Rsrs–Pp			14.395+1.961=16.356
Total	*Dd–Pp*			27.260

[2] S. P. I. denotes accession number of the Division of Plant Exploration and Introduction, formerly Office of Foreign Seed and Plant Introduction.

Unfortunately the seedling albino reported by Karper and Conner (*3*) as linked with seedling stem color, with 41.34 percent crossing over, could not be obtained for studies with this linkage group. The remnant seed was not viable, and probably the character is lost.

INDEPENDENCE OF THE *D Rs P* GROUP FROM CERTAIN OTHER GENES REPORTED

The segregation in F_2 of two or all three members of the linkage group with certain other characters is shown in table 3. The contrasting phenotypes are presence and absence of spreader (*Ss*), colored and white seed (*Rr*), awnless and awned lemmas (*Aa*), normal and antherless flowers (*Alal*), normal and male sterile flowers (*Msms*), and starchy and waxy endosperms (*Wxwx*). The segregations indicate independent inheritance or cross-over values so high that linkage cannot be detected. The inheritance of each of these characters has been reported as being a simple 3 : 1 segregation. Independent segregation of members of this linkage group with members of the *Q B Gs* linkage group was shown in an earlier paper (*13*).

TABLE 3.—*Independent segregation in the F_2 generation of miscellaneous characters with members of the linkage group D (juiciness), Rs (seedling stem color), and P (plant color); counts accumulated over a period of years and from various crosses*

Independent characters	Factors (segregating pairs)	Observed F_2 [1]					χ^2
		Total	*AB*	*Ab*	*aB*	*ab*	
		Number	*Number*	*Number*	*Number*	*Number*	
Spreader	*Ss–Dd*	2,042	1,237	366	333	106	[2] 0.409
	Rsrs–Ss	218	132	39	35	12	.151
Pericarp color	*Rr–Dd*	3,744	2,130	711	690	213	.785
	Rr–Rsrs	2,674	1,529	506	486	153	.177
Awns	*Dd–Aa*	7,165	3,968	1,379	1,332	486	.647
	Rsrs–Aa	3,895	2,163	766	710	256	.064
Antherless	*Aa–Pp*	888	464	168	197	59	1.041
	Dd–Alal	320	189	55	62	14	.413
	Pp–Alal	99	65	10	19	5	.407
Male sterile	*Dd–Msms*	58	35	11	10	2	.572
	Rsrs–Msms	109	57	19	25	8	
	Pp–Msms	377	234	57	74	12	1.598
Waxy	*Dd–Wxwx*	1,822	1,120	286	329	87	.077
	Rsrs–Wxwx	2,202	1,306	331	455	110	.134
	Pp–Wxwx	511	291	79	106	35	.910

[1] Collins' (*1*) formula was used to calculate expected classes.
[2] $n=2$, to obtain P value; $P>0.05$ in each case.

Collins' formula (*1*) was used to calculate expected classes in table 3. P values for deviations from calculated classes are in all cases greater than 0.05.

SUMMARY

This paper reports a linkage group in sorghum of three pairs of genes. The contrasting phenotypes are dry and juicy stalks, red and green seedling stem color, and purple and brown plant color. The factor pairs have been designated *Dd*, *Rsrs*, and *Pp*, respectively, in other papers. Each of these factor pairs shows complete dominance in the F_1 and a 3 : 1 segregation in the F_2. The indicated order of genes, with cross-over percentages, is *D* (10.9) *Rs* (16.4) *P*.

Although in some cases the numbers were small, independent inheritance or cross-over values so high that linkage could not be

demonstrated was found for two or three members of the linkage group with each of the following pairs: Presence and absence of spreader (*Ss*); colored and white seed (*Rr*); awnless and awned lemmas (*Aa*); normal and antherless flowers (*Alal*); normal and male sterile flowers (*Msms*); and starchy and waxy endosperms (*Wxwx*).

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EFFECTS OF NITROGEN COMPOUNDS AND TRACE ELEMENTS ON GROWTH OF ASPERGILLUS NIGER [1]

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INTRODUCTION

A further study of the essentiality of molybdenum (*13*) [2] and gallium (*14*) to *Aspgergillus niger* Van Tiegh. and of the existence of a direct relation between molybdenum and nitrate utilization is reported in this paper.

It is generally accepted that ammonia is the form in which nitrogen serves for the elaboration of organic nitrogen by plants, other forms first undergoing transformation to ammonia. Nevertheless there exist in the literature statements to the contrary, the experimental foundations of which still lack other explanation. Kossowicz (*6*) concluded that when grown on *d*-mannitol and nitrite, *Aspergillus niger* and other fungi utilized nitrite directly without previous reduction to ammonia. The studies of Lemoigne and his coworkers (*7, 8, 9*), on the other hand, have led these investigators to believe that hydroxylamine is the form of inorganic nitrogen converted by the organism into organic nitrogen.

In the present investigation a study was made of growth with practically all the known forms of inorganic nitrogen and with certain simple forms of organic nitrogen. The oversight sometimes made by previous investigators of neglecting to test the nutrient solution, just prior to inoculation, for purely chemical transformations has led to the assumption that all the compounds present were due to biological action. Perhaps the most serious question raised involves the existence in plants of nitrite and hydroxylamine under normal conditions.

The existence of a nitrate-reducing enzyme in plants has been questioned by several investigators and the suggestion has been made that ascorbic acid in the plant serves this function (*8*). Quastel et al. (*12*) report that the ability of bacteria to reduce nitrate is destroyed by heat and acid, conditions not particularly conducive to the destruction of ascorbic acid. Though undeniable evidence of the existence of nitrate reductase is still to be obtained, there is no evidence that ascorbic acid participates in this reduction to any greater extent than other organic materials of the cell of similar capabilities. Mikhlin (*10*) states that reduction of nitrate with ascorbic acid does not proceed to ammonia and that *Chlorella* forms ammonia from nitrite though ascorbic acid appears to be absent. The fact that many organic substances, as well as light and heat, are known to decompose nitrates and nitrites makes tests with the isolated enzyme desirable.

[1] Received for publication August 9, 1939.

[2] Italic numbers in parentheses refer to Literature Cited, p. 747.

REVIEW OF LITERATURE

Since the publication of previous papers by the writer, DeRose et al. (*2*) and Hoagland and Arnon (*5*) have stated that they have found molybdenum essential for green plants. Mulder (*11*) has reported that he was able to verify the necessity of molybdenum for *Aspergillus niger* when nitrogen is supplied as nitrate. Arnon (*1*) found, moreover, that a mixture of elements containing molybdenum but not gallium was beneficial to the growth of barley. Also of interest is the conclusion of Dingle and Sheldon (*3*) that "molybdenum in minute traces is possibly a normal constituent of milk."

EXPERIMENTAL METHODS

The W strain of *Aspergillus niger* was grown in 50 cc. of a 5-percent sucrose solution in 200-cc. pryex flasks for 4 days at 35° C. Information concerning the kind and quantities of mineral salt constituents added to the nutrient solutions is given with the experimental data. The compounds employed included sucrose containing not more than 0.0025 percent of ash, reagent mineral salts, and water redistilled in pyrex glass. Spectroscopically pure salts of the trace elements (Fe, Zn, Cu, Mn, Mo, and Ga) were used in one series of experiments. Sodium hyponitrite and sodium salt of nitrohydroxylaminic acid were prepared and furnished through the courtesy of Dr. Joseph Rosin, of Merck & Co. Other compounds of nitrogen tested are in commercial production. The cultures when harvested were filtered through fritted glass crucibles (IG_3), given a preliminary drying in a current of air at 45°, and dried overnight at 103°. Previous publications by the writer (*13*, *14*) should be consulted for omitted details.

TRACE-ELEMENT EFFECTS WITH VARIOUS NITROGEN SOURCES

SPECIFIC EFFECT OF MOLYBDENUM

The specific effect of molybdenum in the reduction of nitrate nitrogen has been discussed previously (*13*). Exceptionally low yields were obtained when molybdenum was omitted in nutrient solutions containing nitrates of lithium, sodium, potassium, or magnesium. These results have been extended to include the nitrates of calcium, strontium, and barium (table 1).

The toxicity of strontium and barium to *Aspergillus* was either slight or nonexistent in these experiments. The former gave maximum yields, the latter yields that could be increased appreciably, as shown by untabulated data. Cystine hydrochloride was used as a source of sulfur in these experiments to avoid the precipitation of sulfate by strontium and barium.

The specific effect of molybdenum is not unique with nitrates, as formerly assumed, but is also given by nitrites and the sodium salt of nitrohydroxylaminic acid, as described later. Besides demonstrating the ineffectiveness of the cations Ca, Sr, and Ba in influencing results with molybdenum and nitrate, these data emphasize the biological specificity of molybdenum.

TABLE 1.—*Effects of trace-element deficiencies on Aspergillus niger grown with various sources of nitrogen at 35° C. for 4 days* [1]

Element omitted	Calcium nitrate [2]												Strontium nitrate [2]							
	Sample 1				Sample 2				Sample 3				Sample 1				Sample 2			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	82.7	9.02	3.34	4, bl	79.3	8.35	3.17	3, bl	169.0	16.63	3.17	6, bl	167.4	27.02	3.32	10, bl	291.1	31.53	3.24	5, bl
Zn	96.1	10.48	3.09	4, bl	35.1	3.70	2.99	1, bl	97.8	9.62	3.04	6, bl	123.9	20.00	3.16	8, bl	44.3	4.80	3.28	2, bl
Cu	866.6	94.50	3.23	2, bl	955.9	100.69	2.48	5, t	978.5	96.30	2.67	4, t	823.9	132.97	2.38	4, t	935.6	101.33	3.15	4, w
Mn	547.8	59.73	3.35	0	986.0	103.87	2.47	5, bl	885.0	87.11	2.16	0	710.0	114.59	2.04	0	1,013.3	109.75	2.40	0
Mo	891.6	97.23	2.91	8, bl	99.3	10.46	3.36	4, bl	826.9	81.39	2.88	8, bl	663.4	107.07	2.55	10, bl	150.5	16.30	3.40	3, bl
None	917.0	100.00	3.59	8, bl	949.3	100.00	3.19	8, bl	1,016.0	100.00	3.04	8, bl	619.6	100.00	2.94	10, bl	923.3	100.00	3.16	------
Maximum [5]	1,130.0	------	------	------	1,190.3	------	------	------	1,183.2	------	------	------	868.3	------	------	------	1,193.8	------	------	------
C. U. [6]	------	44.52	------	------	------	43.63	------	------	------	47.33	------	------	------	34.73	------	------	------	47.75	------	------
pH [7]	------	------	4.63	------	------	------	4.66	------	------	------	4.71	------	------	------	3.09	------	------	------	3.49	------

See footnotes at end of table.

TABLE 1.—*Effects of trace-element deficiencies on Aspergillus niger grown with various sources of nitrogen at 35° C. for 4 days* [1]—Continued

Element omitted	Strontium nitrate [2] Sample 3				Barium nitrate				Sodium nitrite				Hydroxylamine [3] hydrochloride				Sodium salt of nitrohydroxylaminic acid			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [4]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	456.3	42.24	3.28	8, bl	233.4	30.62	2.50	2, bl	290.6	63.93	3.15	8, bl	98.9	72.99	2.36	6, bl	150.3	32.17	3.01	4, bl
Zn	44.9	4.15	3.27	2, bl	129.9	17.05	3.06	10, bl	99.0	21.78	3.14	6, bl	90.2	66.56	2.38	6, bl	238.7	51.09	3.28	1, bl
Cu	1,106.9	102.46	2.84	4, t	508.1	66.66	3.25	0	477.3	105.02	2.64	10, t	102.9	75.93	2.35	6, bl	463.2	99.15	3.59	4, br
Mn	1,068.3	98.88	2.32	0	424.4	55.68	2.79	0	486.3	107.00	2.86	10, bl	126.6	93.43	2.29	6, bl	506.3	108.37	2.90	0
Mo	1,078.4	99.82	2.94	8, bl	69.7	9.14	2.85	0	74.6	16.41	3.13	4, bl	148.4	109.52	2.28	6, bl	150.9	32.29	2.73	2, bl
None	1,080.3	100.00	3.02	8, bl	762.3	100.00	2.89	8, bl	454.5	100.00	3.02	10, bl	135.5	100.00	2.33	6, bl	467.2	100.00	3.58	8, bl
Maximum [5]	1,185.3				945.8				495.0				168.0				514.9			
C. U. [6]		47.41				37.83				19.80				6.72				20.60		
pH [7]			3.46				2.97				3.75				4.21				2.19	

[1] Nitrogen was used at a concentration of 720 mg. per liter, except sodium nitrite at 2.0 gm., hydroxylamine hydrochloride at 0.5 gm., and sodium salt of nitrohydroxylaminic acid at 1.57 gm. per liter. Other constituents were sucrose at 50 gm. per liter; KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ at 0.55 gm. and 0.25 gm. per liter respectively; and Fe, Zn, Cu, Mn, and Mo, at 0.20, 0.20, 0.05, 0.02, 0.02 mg. per liter respectively. Cystine hydrochloride was substituted for sulfate ion in equivalent amount in the experiments with strontium and barium nitrates.

[2] Samples from different manufacturers.

[3] Fungus harvested after 22 days.

[4] Sporulation is indicated on a scale of 0 (sterile) to 10 (black with spores), and spore color by the initial letter or first 2 letters of the words white, yellow, tan, brown, and black.

[5] Maximum individual yield.

[6] Coefficient of utilization, or yield per 100 gm. of sucrose.

[7] Initial acidity of the culture solution.

VARYING TRACE-ELEMENT CONTENT OF NITRATES

Table 1 shows also the wide variation in response to trace-element deficiencies with salts obtained from different manufacturers. The percentages of maximum yield obtained with calcium nitrate varied over 40 percent in minus-manganese cultures and nearly 87 percent in minus-molybdenum cultures. Data for still another sample of calcium nitrate will be found in the article (*13*) last mentioned. A similar marked variation in percentage of maximum yield in minus-molybdenum cultures was obtained with strontium nitrate.

RELATIVE VALUE OF NITROGEN SOURCES

Hydroxylamine proved a poor source of nitrogen. Sodium nitrite can also be so characterized, since chemical tests indicated that at the acidity necessary for growth decomposition occurred even prior to sterilization. A decided odor of nitric oxide could be detected, and tests revealed the presence of nitrate, hydroxylamine, ammonia, and residual nitrite. A somewhat similar situation exists in the utilization of potassium cyanate (see table 3), since at an acidity sufficient to permit growth complete destruction of cyanate with concomitant formation of ammonia had taken place. The effects of molybdenum deficiency with nitrite and with nitrohydroxylaminic acid salt indicate quite clearly that this element continues to function in the further reduction of these reduction products of nitrate.

YIELDS IN OPTIMUM SOLUTIONS CONTAINING GALLIUM

In the experiments of table 2 spectroscopically pure, instead of reagent, trace elements were used, and the data were extended to include the effects of a deficiency of gallium. Sucrose of 0.0014 percent ash extracted with 95-percent alcohol was employed. While these experiments were effective in demonstrating the need for gallium by *Aspergillus niger* under a variety of conditions in pyrex glassware and of the suitability of various procedures for such tests, the specific molybdenum-nitrate response was no longer apparent. It seems probable, therefore, that the solution of spectroscopically pure gallium used contained a minute trace of molybdenum. Though this interpretation may seem farfetched, the opposite is true. No molybdenum, for example, could be found in the ash of the mycelial felts nor in the reagents with a Bausch and Lomb large-size quartz spectrograph by Dr. B. C. Brunstetter, of the Bureau of Plant Industry. Similar tests on reagents by Dr. Joseph Rosin, of Merck & Co., were also negative, and were attributed by him to the lack of sensitivity of the Bausch and Lomb medium-size quartz spectrograph used. His estimate that double the sensitivity would be required to detect the presence of molybdenum in the reagents is in all probability too low. A small molybdenum deficiency is shown in each of the solutions, but one corresponding only to a maximum deficiency measured as loss in yield of 27 percent instead of 90 percent. The assumed trace of molybdenum in the gallium solution, of course, supplements that added with the other constituents of the solution.

TABLE 2.—*Effects of trace-element deficiencies, particularly gallium, on Aspergillus niger grown with various nitrogen sources at 35° C. for 4 days* [1]

Element omitted	NH_4NO_3				NH_4Cl				$NaNO_3$				Urea			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	220.3	25.29	2.00	6, bl	541.5	51.66	1.66	6, bl	298.3	31.17	3.25	8, bl	355.1	31.20	2.49	8, bl
Zn	35.8	4.11	2.76	6, bl	33.1	3.16	2.69	4, bl	22.9	2.39	3.23	2, bl	39.8	3.50	2.74	2, bl
Cu	567.2	65.12	1.85	6, bl	870.4	83.05	1.47	4, bl	933.6	97.56	2.68	6, br	1,096.4	96.33	2.22	6, br
Mn	484.5	55.63	1.79	0	623.3	59.47	1.51	0	821.4	85.84	2.40	8, bl	1,045.1	91.82	2.09	6, bl
Mo	674.6	77.45	1.88	2, bl	802.9	76.61	1.45	4, bl	694.7	72.60	3.09	8, bl	976.4	85.79	2.06	10, bl
Ga	594.0	68.20	1.88	4, bl	958.9	91.49	1.45	4, bl	743.0	77.65	3.15	8, bl	598.6	52.59	2.27	10, bl
None	871.0	100.00	1.90	4, bl	1,048.1	100.00	1.46	4, bl	956.9	100.00	2.49	10, bl	1,138.2	100.00	2.31	10, bl
Maximum [4]	941.6				1,091.3				975.1				1,169.9			
C. U. [5]		37.66				43.65				39.00				46.68		
pH [6]			7.26				7.22				7.59				7.64	

Element omitted	NH_4NO_3 (purified with CaO) [3]				NH_4Cl (purified with CaO) [3]				$NaNO_3$ (purified with CaO) [3]				Urea (purified with CaO) [3]			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	18.3	1.78	2.63	1, bl	16.0	1.53	2.77	1, bl	14.4	1.75	2.71	1, bl	21.9	2.72	2.81	0, bl
Zn	22.8	2.22	2.80	1, bl	32.4	3.10	2.74	1, bl	21.5	2.62	3.26	1, bl	44.7	5.56	2.89	0, bl
Cu	864.4	84.15	2.04	4, br	883.0	84.53	1.46	3, bl	871.0	106.27	2.77	10, t	633.7	78.85	2.79	3, br
Mn	300.5	29.25	1.76	0	514.5	49.26	1.60	0	606.9	74.04	1.87	0	164.2	20.43	3.19	0
Mo	900.3	87.66	2.00	2, bl	791.1	75.73	1.47	2, bl	709.4	86.55	2.70	10, bl	649.6	80.82	2.93	2, bl
Ga	565.5	55.06	1.78	6, br	910.2	87.13	1.42	2, bl	462.6	56.44	3.39	8, bl	421.7	52.47	2.87	1, bl
None	1,027.1	100.00	1.99	4, bl	1,044.6	100.00	1.43	4, bl	819.7	100.00	2.63	10, bl	803.7	100.00	2.18	8, bl
Maximum [4]	1,070.3				1,121.0				896.5				989.2			
C. U. [5]		42.81				44.84				35.86				39.57		
pH [6]			7.44				7.31				7.22				7.27	

[1] See footnote 1, table 1.
[2] Somewhat higher concentrations of constituents were used to avoid non-trace-element deficiencies.
[3] See footnote 4, table 1.
[4] See footnote 5, table 1.
[5] See footnote 6, table 1.
[6] See footnote 7, table 1.

NUTRITIVE VALUE OF SOME FORMS OF NITROGEN

The experiments shown in table 3, p. 738, are largely a repetition of those shown in table 1, except that gallium was added in each case and an experiment with potassium cyanate (KCNO) was included. The latter experiment has been discussed in connection with table 1. It will be noted that the results with molybdenum deficiency are quite poor when nitrogen is supplied as nitrohydroxylaminic acid, but that they are somewhat better with nitrogen as nitrite. These results are attributed to the cumulative effect of molybdenum impurity and not to any difference in behavior of these types of nitrogen. Of the intermediate products of nitrate reduction—nitrous acid (HNO_2), nitrohydroxylaminic acid ($H_2N_2O_3$), hyponitrous acid ($H_2N_2O_2$), and hydroxylamine (NH_2OH)—nitrohydroxylaminic acid and hyponitrous acid proved least toxic. The former ($H_2N_2O_3$) was the better source of nitrogen for growth of the fungus, the latter ($H_2N_2O_2$) could not be utilized. Nitrohydroxylaminic acid, like nitrous acid, was found to give a positive test with potassium iodide-starch, Griess reagent, and diphenylamine. The iodine-Griess reaction, or Blom test, for hydroxylamine is also given by nitrohydroxylaminic acid. Though the sodium salt of hyponitrous acid ($Na_2N_2O_2$) did not support growth, it permitted excellent germination to take place. The addition after several weeks of a favorable nitrogen source or the transfer of a bit of the submerged mycelim to Czapek agar resulted in rapid and normal growth.

Because of their marked toxicity, the quantity of sodium nitrite was limited to 2.0 gm. per liter and that of hydroxylamine hydrochloride to 0.5 gm. per liter. Sodium salt of nitrohydroxylaminic acid, and sodium hyponitrite were employed in amounts equivalent to 360 mg. of nitrogen per liter. Use of higher concentrations of these two salts was practicable, but the quantity of available material was small. All the other forms of nitrogen were used in quantities corresponding to 720 mg. of nitrogen per liter. The length of the growth period was 4 days with readily available sources of nitrogen but was extended sufficiently to obtain approximately maximum growth with other nitrogen sources.

GROWTH ON *d*-MANNITOL

Growth on *d*-mannitol (table 4) was uniformly less than with sucrose. Response of the organism, however, on this carbon source was quite similar to that on sucrose, both with trace elements and with different sources of nitrogen. The sample of mannitol employed was apparently not so free from trace-element impurities as the sucrose ordinarily employed, judging from the deficiency results obtained. Nevertheless, the same trace elements were required with both sources of carbon. A definite difference seemed to exist, however, in the production of organic acids inasmuch as final acidities at harvest were comparatively low with mannitol. The high acidities with ammonium chloride are laid to the effect of residual chloride ion.

TABLE 3.—*Effects of trace-element deficiencies and oxidative level of nitrogen supply on the growth of Aspergillus niger grown at 35° C. for 4 days* [1]

Element omitted	Sodium nitrite				Sodium salt of nitrohydroxylaminic acid [2]				Sodium salt of nitrohydroxylaminic acid [3]			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [5]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [5]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [5]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	148.3	34.95	3.06	6, bl	261.5	90.79	3.09	8, bl	269.0	75.33	3.17	8, bl
Zn	20.2	4.75	3.28	2, bl	261.8	90.90	3.07	8, bl	384.5	107.67	3.14	4, bl
Cu	432.6	101.95	2.71	10, br	358.3	124.41	3.11	8, bl	365.2	102.27	3.25	8, bl
Mn	541.3	127.57	2.45	8, bl	3.7	1.28	5.09	8, bl	549.0	153.74	3.47	8, br
Mo	191.8	45.21	3.03	8, bl	268.0	93.06	2.77	0	279.0	78.13	3.35	8, bl
Ga	410.6	96.77	3.03	10, bl	360.6	125.21	3.32	8, bl	359.3	100.61	3.43	8, bl
None	424.3	100.00	2.97	10, bl	288.0	100.00	3.09	8, bl	357.1	100.00	3.26	8, bl
Maximum [6]	541.3				360.6				549.0			
C. U. [7]		21.65				14.42				21.96		
pH [8]			4.15				8.32				5.76	

Element omitted	Sodium salt of nitrohydroxylaminic acid				Hydroxylamine hydrochloride [4]				Potassium cyanate			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [5]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [5]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation [5]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	168.9	60.93	2.98	4, bl	107.4	76.55	2.24	6, bl	474.3	42.90	2.23	8, bl
Zn	177.7	64.10	2.98	4, bl	68.2	48.61	2.34	6, bl	710.2	64.24	1.94	10, bl
Cu	331.3	119.52	2.92	6, bl	134.8	96.08	2.22	6, bl	1,117.3	101.06	1.87	2, w
Mn	358.8	129.43	2.77	8, bl	119.9	85.46	2.13	1, bl	669.7	60.58	1.86	0
Mo	305.9	110.36	2.89	8, bl	158.3	112.83	2.20	6, bl	1,142.2	103.31	1.86	10, bl
Ga	268.3	96.78	2.94	8, bl	138.8	98.93	2.24	6, bl	1,131.5	102.35	1.86	10, bl
None	277.2	100.00	2.96	8, bl	140.3	100.00	2.22	6, bl	1,105.6	100.00	1.86	10, bl
Maximum [6]	358.8				158.3				1,146.8			
C. U. [7]		14.35				6.33				45.87		
pH [8]			4.24				2.97				7.26	

[1] See footnote 1, table 1.
[2] Fungus harvested after 19 days.
[3] Fungus harvested after 20 days.
[4] Fungus harvested after 21 days.
[5] See footnote 4, table 1.
[6] See footnote 5, table 1.
[7] See footnote 6, table 1.
[8] See footnote 7, table 1.

Chemical tests for inorganic nitrogen compounds in solution revealed that mannitol differed from sucrose in other ways besides being a poorer medium for growth. These differences were most marked with nitrite. Only minute traces of ammonia could be detected in the culture solutions as a rule, and the Griess test for nitrite was usually negative until some time had elapsed, when a strong reaction might be obtained. Ammonium nitrate cultures seemed free of nitrogen except for faint traces of ammonia and nitrite which increased in intensity with duration. The absence of ammonia in the cultures could of course be due to the direct utilization of nitrite for formation of organic nitrogen as postulated by Kossowicz (*6*). In view of the marked toxicity of nitrite, a more logical interpretation is that with retardation of acid production the reduction to ammonia proceeds so slowly that little or none accumulates under ordinary conditions. The decided lag in tests for inorganic nitrogen may be indicative of combination with mannitol. Another possibility lies in the existence of still other reduction products as yet unknown. Nevertheless ammonia with mannitol, as with sucrose, is a far better source of nitrogen than is nitrite.

TABLE 4.—*Effects of trace-element deficiencies and source of nitrogen on Aspergillus niger grown on d-mannitol at 35° C.*[1]

Element omitted	Growth on *d*-mannitol with—																			
	NH_4Cl (8 days)				$NaNO_3$ (7 days)				$NaNO_3$ (19 days)				$NaNO_3$ (21 days)				NH_4NO_3 (7 days)			
	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. of sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	418.1	79.88	1.63	2, bl	318.3	67.26	5.83	8, bl	41.7	249.70	6.53	2, bl	99.2	45.11	6.98	[3]6	230.3	26.16	2.00	6, b
Zn	175.8	33.58	2.06	4, bl	90.4	19.10	2.47	2, bl	15.0	89.82	6.55	2, bl	129.9	59.08	5.15	[3]6	185.2	21.03	2.21	6, b
Cu	453.1	86.56	1.48	2, bl	562.0	118.76	4.15	6, t	19.7	117.96	6.37	2, br	426.0	193.72	5.22	[3]6	798.4	90.67	3.23	4, bl
Mn	608.7	116.30	1.41	2, bl	305.8	64.62	4.64	6, bl	26.5	158.68	6.82	2, bl	598.3	272.07	4.17	[3]6	490.1	55.66	2.01	2, bl
Mo	591.0	112.92	1.38	2, bl	444.1	93.85	4.91	10, bl	6.1	36.52	6.60	1, w	202.0	91.86	6.67	[3]6	531.2	60.33	1.89	8, bl
Ga	464.8	88.80	1.42	2, bl	537.3	113.55	6.32	10, bl	9.1	54.49	7.08	2, br	315.5	143.47	7.35	[3]6	735.7	83.56	2.76	4, bl
None	523.4	100.00	1.47	2, bl	473.2	100.00	5.32	8, bl	16.7	100.00	6.46	2, br	219.9	100.00	7.31	[3]6	880.5	100.00	3.97	6, bl
Maximum[4]	608.7				573.5				41.7				598.3				913.1			
C. U.[5]		34.35				22.94				1.67				23.93				36.52		
pH[6]			4.03				2.24				7.59				4.03				3.92	

[1] See footnote 1, table 1. Mannitol at 50 gm. per liter was used instead of sucrose.
[2] See footnote 4, table 1.
[3] Spores yellow to black.
[4] See footnote 5, table 1.
[5] See footnote 6, table 1.
[6] See footnote 7, table 1.

REDUCTION PRODUCTS OF NITRATE

COMPOUNDS OF NITROGEN WITH HYDROGEN OR OXYGEN

There are 19 known compounds of nitrogen containing only hydrogen or oxygen or both. They include 5 compounds generally considered to be reduction products of nitric acid, 8 oxides, hydrazine, hydrazoic acid, nitramide, pernitric acid, and hydronitrous acid. The 5 members of the nitrate reduction series are nitrous acid (HNO_2), nitrohydroxylaminic acid ($H_2N_2O_3$), hyponitrous acid ($H_2N_2O_2$), hydroxylamine (NH_2OH), and ammonia. Growth of *Aspergillus* was tested with all of the 19 compounds except 7 of the oxides (N_2O was included), nitramide, pernitric acid, and hydronitrous acid. Since the oxides of nitrogen other than nitrous oxide decompose in aqueous solution with formation of members of the nitrate reduction series, their omission was unimportant. Nitramide could not be obtained, since it undergoes violent decomposition at 72° C.; pernitric acid as an oxidation product of nitric acid was unnecessary; and hydronitrous acid does not exist in the presence of water. The nitrogen compounds included are considered, therefore, to comprise a complete list of the known possibilities. A number of organic nitrogen compounds were also included in these tests.

ASSIMILABILITY OF NITRATE-AMMONIA SERIES

The members of the nitrate-ammonia series differ widely in their acceptability to *Aspergillus* as a source of nitrogen. Nitrates, ammonium salts, and sodium salt of nitrohydroxylaminic acid provide excellent nontoxic sources of nitrogen available over a wide range in acidity, whereas nitrite and hydroxylamine are quite toxic and practically useless for growth. The reason for characterizing nitrite as useless is that at an acidity permitting growth the nitrite is decomposed with the formation of nitrate and ammonia. Growth does not take place on nitrite if the solution is neutral or only slightly acid. Hydroxylamine is also quite toxic and could not be used in concentrations over 0.5 gm. per liter, and requires markedly acid solutions to permit slight growth. Spore germination in cultures with sodium hyponitrite is excellent, but practically no growth takes place because, although the hyponitrite is nontoxic, its nitrogen is unavailable.

NITROGEN COMPOUNDS IN NUTRIENT SOLUTIONS

The precaution was taken of testing the various nutrient solutions for the presence of the different forms of inorganic nitrogen. The chemical tests that were used are shown in table 5, and were verified with the nitrogen compounds as soon as they were received. It is important to note, particularly, that nitrohydroxylaminic acid gives every positive test given by nitrous acid, besides giving a positive iodine-Griess, or Blom test. Boiling the solution with sulfanilic acid to destroy nitrite evidently results in the production of some hydroxylamine from nitrohydroxylaminic acid, since a positive Griess test is given by its subsequent oxidation to nitrite with iodine.

TABLE 5.—*Qualitative tests for some forms of inorganic nitrogen in dilute solution* [1]

Nitrogen compound [2]	Reaction with—				
	Starch-potassium iodide	Griess nitrite test	Diphenyl-amine	Iodine-Griess test [3]	Nessler reagent
Nitrate	−	−	+	−	−
Nitrite	+	+	+	−	−
Salt of nitrohydroxylaminic acid	+	+	+	+	−
Hyponitrite	[4] ±	[5] −	−	+	−
Hydroxylamine	−	−	−	+	+
Ammonia	−	−	−	−	+

[1] The procedures used in making these tests are described in Fred and Waksman's manual (4). A positive reaction is indicated by +, a negative by −. The salts were tested immediately upon receipt.
[2] The gases NO_2 and NO are reported to give positive starch-iodide and Griess tests.
[3] Iodine is used to oxidize hydroxylamine, etc., to nitrite before testing.
[4] Usually stated to be negative; test faint but definite.
[5] Usually stated to be positive.

A positive Griess test indicates the presence of either nitrous acid or nitrohydroxylaminic acid; a positive diphenylamine-sulfuric acid test may be due to the presence of nitric, nitrous, or nitrohydroxylaminic acids; and a positive Blom test indicates the presence of nitrohydroxylaminic acid, hyponitrous acid, or hydroxylamine. The positive ammonium test with hydroxylamine is probably due to the presence of decomposition products, since hydroxylamine is quite unstable.

The results obtained with these chemical tests of nutrient solutions just prior to inoculation are given in table 6. The nonspecificity of these tests should be borne in mind. It is clear, however, that some decomposition of these nitrogen compounds had taken place in culture solution before inoculation and that the products differed with the acidity. A possible connection may exist between the faculty of preinoculation formation of ammonia and availability of the compound for growth, since hyponitrous acid shows no trace of ammonia formation nor does nitrous acid in neutral solution. The nitrogen compounds identified after growth has taken place may therefore not always be due to biological action, as some investigators have assumed.

TABLE 6.—*Preinoculation nitrogen decomposition products in nutrient solutions containing sucrose* [1]

Nitrogen compound added	Preinoculation decomposition products in acid solution	Preinoculation decomposition products in alkaline or neutral solution	Supplementary nitrogen compounds aiding growth
Nitrate		HNO_2, (NH_2?)	
Nitrite	HNO_2, NH_3	HNO_2, NH_2OH	None.
Nitrohydroxylaminic acid salt	HNO_2, NH_3	HNO_2, HNO_2, NH_2OH	NH_2OH (slight).
Hyponitrite [2]	HNO_2, NH_2OH	(HNO_2?), (NH_2OH?)	NH_2, HNO_2, urea, $H_2N_2O_2$.
Hydroxylamine	NH_3	NH_3	NH_2.
Ammonia			

[1] The tabulated results will probably differ under different conditions.
[2] Addition of $\frac{N}{10}$ NaOH previous to Nessler test resulted in a strong ammonia reaction.

The last column of table 6 indicates the compounds of nitrogen that serve to increase yield when added to approximately neutral nutrient solutions provided with nitrogen of the form stated in the first column.

An attempt has been made to distinguish between the supplementary nitrogen compounds that bring about a rapid increase in the rate of growth and those causing a final increase without markedly influencing the rate. It is those falling within the former group only that are listed, since inclusion of the latter group would, except with nitrite, necessitate the addition of practically all nitrogen compounds tested. The rate of growth is maximum with ammonium and nitrate nitrogen and these were not tested. Toxicity is probably the cause of the negative results with nitrite, just as only nitrogen deficiency with hyponitrite is the reason for positive results with most of the compounds tested. The beneficial action of hydroxylamine hydrochloride on growth with sodium salt of nitrohydroxylaminic acid is attributed to the marked acidity of the hydrochloride. Some chemical interaction may be responsible, however. Hydroxylamine, therefore, was the only nitrogen source showing a definite response when supplemented with another source of nitrogen (NH_3), which seems to indicate that reduction and not oxidation is most beneficial in improving the assimilability of hydroxylamine by *Aspergillus*.

The state of reduction of inorganic nitrogen would seem to be without intrinsic effect upon the formation of mass per unit of nitrogen supplied, a result that also seems to indicate that these various compounds are transformed into a single compound for elaboration into organic nitrogen. If the maximum yield per milligram of nitrogen supplied is computed it is found that nitrate, ammonium, and hydroxylamine form approximately 33 mg. of mass. With nitrite the fungus formed 30 mg. of mass per milligram of nitrogen, and with nitrohydroxylaminic acid approximately 31 mg. of mass. The slightly lower yields may be due to experimental error or to chemical side reactions.

Hydrazine sulfate ($N_2H_4.H_2SO_4$) and sodium azide (N_3Na) were unsuitable for supplying nitrogen in culture, except as supplementary sources of nitrogen. Nitrous oxide also failed to support growth. The following organic substances were tested and found to give little growth: *n*-heptaldoxime, acetone oxime, dimethylglyoxime, urethane, methylurea, phenylurea, diazoaminobenzene, azobenzene, and sulfamic acid. No germination occurred with *s*-diphenylurea, azoxybenzene, hydrazobenzene, and methylhydrazine sulfate. Therefore, the attempts to supply the fungus with sufficient hydroxylamine at low concentrations for maximum growth by using difficultly soluble oximes that might furnish this compound through slow hydrolysis proved unsuccessful. Moreover, the tests would seem to indicate that substitution of organic radicals for hydrogen in urea and in the members of the nitrate reduction series practically prevents their use as a source of nitrogen. Neither did substitution of radicals in hydrazine and hyponitrous acid lead to any improvement in nutritiveness.

POST-GROWTH METABOLIC PRODUCTS IN CULTURE

CONSTITUENTS FOUND

The tests shown in table 5 were also employed to identify inorganic nitrogen compounds in solution at the time of harvest. In addition, urea was sought for with xanthydrol and the presence of soluble amino nitrogen compounds with phosphotungstic acid containing 5 percent

of sulfuric acid. Also included were tests for starch in both mycelial felt and culture solution. It is not known what substances are responsible for the results of the ferric chloride test. All reactions were carried out on porcelain test plates with about 0.5 ml. of solution, and were essentially qualitative. The quantities of precipitate and degrees of coloration afforded a rough measure of quantities concerned, however. The results obtained will be found in table 7.

In control cultures with any source of nitrogen suitable for growth there were usually found various derived nitrogen compounds, usually, with one exception, reduction products. However, hydroxylamine was formed from ammonium salts with sucrose but not with *d*-mannitol. The formation of urea and of soluble amino acids or their condensation products was also general. A marked difference in starch (and amino acid) formation was found between solutions with ammonium and nitrate nitrogen in that starch was formed profusely with ammonium, whereas little or no starch was formed with nitrate. The starch responses with urea and potassium cyanate resembled those with ammonium, whereas with nitrate, nitrohydroxylaminic acid, and hydroxylamine these responses resembled those with nitrate. Acidity was an important factor in starch formation also.

EFFECTS OF TRACE-ELEMENT DEFICIENCIES

The effects of a trace-element deficiency were not always the same, neither was the extent of the deficiency on yield. Generally, the greater the deficiency the smaller were the quantities of products formed and the greater were the concentrations of residual ions initially added. Low yields because of iron deficiency were frequently accompanied by an increased hydroxylamine production, whereas low zinc prevented its formation. A phenomenon that was noticed repeatedly, but not invariably, was associated with molybdenum deficiency in ammonium nitrate solutions containing gallium. A low yield (73 percent or less) was accompanied in these cases by high acidity and profuse starch formation, the residual ammonia being relatively low and the residual nitrate high. The data in table 7 do not show this clearly, but the dibasic optimum solution purified with di- or tri-calcium phosphate can generally be depended upon to give this response. The increased acidity in minus-molybdenum cultures may possibly contribute to the increase in starch formation, but does not seem to effect reduction of nitrate to any extent. Manganese deficiency leads to similar responses that differ only in the formation of an immediate brilliant-blue test for starch in the felts, whereas low molybdenum gives a brown iodine test with the mycelial felt that changes to a deep blue eventually. Though these may be wholly nonspecific deficiency responses, the very fact that omission of an element causes such a response would afford additional evidence of the essentiality of that element to growth.

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TABLE 7.—*Results of tests for various metabolic products of Aspergillus niger in solution at time of harvest* [1]

| Constituent found in cultures at time of harvest | Element omitted from solution with— | | |

DISCUSSION

The necessity of molybdenum for the growth of *Aspergillus* is made still more certain by the markedly poor growth of the fungus when this element has not been added to nutrient solutions containing sodium nitrite and sodium salt of nitrohydroxylaminic acid. It may be assumed, therefore, that molybdenum is necessary for the reduction of these compounds as well as of nitrate. The absence of positive results with sodium hyponitrite and with hydroxylamine requires further elucidation, however. The effect of molybdenum deficiency with ammonium nitrate is assumed to depend on the "locking" of nitrate, so that the organism is actually growing in effect with ammonium nitrogen. Further chemical work of a quantitative nature will be necessary for a complete understanding of the processes involved. The very existence of the molybdenum-nitrate effect would indicate the essentiality of this element for growth. The diminished effect of withholding molybdenum when gallium is added is not attributed to gallium but to the presence of impurities. Other experiments not reported here have resulted in diminutions in yield of more than 60 percent.

The data on gallium deficiency contain nothing intrinsically new, since they are but an extension and amplification of the data formerly reported (*11*). The nonaddition of gallium to the nutrient solution resulted in a lessened growth in certain instances, but the effects were slight except under the best of conditions, namely, the experiments in table 2, in which sucrose extracted with 95-percent alcohol was used.

The results of the experiments with the reduction products of nitric acid are taken to indicate that ammonium is the only source of inorganic nitrogen of direct service to the fungus for the elaboration of organic nitrogen. Other sources of nitrogen are converted into ammonia before use, and their nutritiveness would seem proportional to the rate and extent of their conversion.

Tests with mixed nitrogen sources were made on the assumption that rate of formation of reduction or oxidation products in the nitrate-ammonium series depends on initial concentration of the compound undergoing reaction and the rate of removal of the final product (law of mass action). Within the limits fixed by the requirements of the organism only the addition of ammonia to hydroxylamine proved effective. Since hydroxylamine was employed at the maximum concentration its toxicity permitted, the increased yield cannot be attributed to an increase in hydroxylamine concentration through oxidation of ammonia. It is considered, on the contrary, as a demonstration that hydroxylamine is employed by the fungus only after reduction to ammonia.

The association between assimilability and decomposition with acid to form ammonium with the reduction products of nitrate would seem to have a similar interpretation. The poor growth with nitrite at low acidities is no exception if the toxicity of nitrite be considered. At high acidity, moreover, nitrite is also decomposed with the formation of ammonium. The only evidence obtained for the formation of ammonium from hyponitrite was under alkaline conditions entirely unsuitable for growth of the fungus. However, these compounds are highly unstable, decomposition sometimes proceeding at an explosive rate, and the products formed depend largely on the conditions of the experiment.

Acid decomposition of nitrous and nitrohydroxylaminic acids probably is not the primary reaction employed for the production of ammonia by the organism, since growth in acid solution is quite meager in the absence of molybdenum. Nevertheless proof that molybdenum is not concerned in the purely chemical production of ammonia by acid in the nutrient solution would strengthen the interpretation that these reduction processes are really enzymatic.

The considerable evidence that has accumulated for the existence of nitrite and hydroxylamine in the tissues of green plants and microorganisms would seem to require further verification in the light of these results. The data regarding these compounds are more logically explained on the assumption of the presence of nitrohydroxylaminic acid. This compound is also a reduction product of nitrate and is, above all, of little toxicity. The presence of hydroxylamine among its decomposition products formed by acidity would readily account for the identification of this relatively toxic compound in the tissues of organisms.

The experimental results of Kossowicz (*6*) with nitrite nitrogen and *d*-mannitol were duplicated. They cannot be accepted as evidence for the direct utilization of nitrite nitrogen for several reasons, however. Nitrites are quite as toxic with mannitol as with sucrose. A slower rate of reduction to ammonia with mannitol could also serve to explain these experimental results, especially if combined with the failure of growth to cause a normal increase in acidity of the cultures. The consistent presence of traces of ammonia in the cultures renders that interpretation not improbable. There is, in addition, the possibility that chemical combination occurs between mannitol and nitrite at least, since the Griess test is long delayed. Similar lags in response were obtained with both the ammonia and nitrite tests when ammonium nitrate was the source of nitrogen.

The main objections to be met before the view can be accepted that hydroxylamine is the primary source of inorganic nitrogen would seem to be those based on its toxicity and slow assimilation. Its formation from inorganic nitrogen of higher oxidation level is to be more or less expected, but this in itself is no evidence of its utilization in this form. Its presence in solution in certain instances may be and probably is in the role of a waste product usually in association with high ammonium concentration. The formation of hydroxylamine from ammonium chloride must be interpreted upon some such basis. Oxidation of ammonium to hydroxylamine in the culture solution is questionable, since its reduction potential is sufficient for formation of ammonium. Molybdate is reduced to "molybdenum blue" and methylene blue becomes green.

The attractiveness of the hydroxylamine hypothesis consists in the ease with which α-ketonic acids may be converted to amino acids through oxime formation with hydroxylamine, and their subsequent reduction to amino acid and regenerated hydroxylamine. Aminization of α-ketonic acids is also readily accomplished, however, by Knoop's method of catalytic reduction in the presence of ammonia. Additional evidence would be desirable, however, on the extent to which degradation of hexoses by organisms results in the formation of α-ketonic acids. Mammalian forms have been claimed to possess to a limited degree the ability to form amino acid from ammonium salts.

Repetition of some phases of these studies from the chemical viewpoint would seem desirable. Development of specific qualitative and quantitative methods would be of paramount importance, however. *Aspergillus*, in itself and because of its close correspondence to green plants as regards nitrogen utilization, furnishes excellent material for such investigations.

SUMMARY

Growth of *Aspergillus niger* in culture solution has led to additional proof of the essentiality of iron, zinc, copper, manganese, and particularly molybdenum and gallium. Molybdenum seems to be of special importance in the reduction not only of nitrates but of nitrites and nitrohydroxylaminic acid salt as well.

Data are presented to indicate the wide variation in trace-element content of different samples of reagent nitrates. Nitrates, ammonium salts, and nitrohydroxylaminic acid salt were found to be the best sources of inorganic nitrogen for growth. Nitrite, hyponitrite, hydrazine, azide, and nitrous oxide were useless for growth. Hydroxylamine was a poor source of nitrogen. The positive tests for nitrite in organisms are attributed to nitrohydroxylaminic acid, whose presence may also account for the hydroxylamine reported to be present.

Analogies were found between the ability of inorganic nitrogen compounds to form ammonia chemically, under the influence of acidity, and their assimilability. Substitutions of organic radicals in such compounds in no case improved assimilability. Ammonia is concluded to be the primary source of nitrogen for conversion to organic nitrogen.

Specific starch reactions associated with manganese and molybdenum deficiency are described as a phenomenon repeatedly noticed in the formation of post-growth metabolic products in culture.

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RELATION OF CARBON NUTRITION TO TRACE-ELEMENT AND ACCESSORY REQUIREMENTS OF ASPERGILLUS NIGER [1]

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INTRODUCTION

Previous investigations by the writer (*18*) [2] on trace-element and accessory-factor needs of *Aspergillus niger* Van Tiegh. have been limited to nutrient solutions containing sucrose. Studies with other sources of carbon were considered desirable, therefore, for several reasons. The nature of impurities in different carbon compounds might vary sufficiently to give improved deficiency tests with some of the essential trace elements. On the other hand, the possibility existed that the trace-element requirements of the organism might vary quantitatively with modification in nutrition, as in the case of molybdenum and nitrate (*16*). Lastly, there was a further possibility that glycerol might prove an effective substitute for sucrose in the metabolism of the fungus and that it might be feasible to obtain it in a higher state of purity than sucrose by repeated distillation in the laboratory.

The present paper reports results obtained with glycerol and with various other carbon compounds.

REVIEW OF LITERATURE

A recent review of the literature has been published elsewhere by the writer (*18*), and only investigations of direct importance to these studies need be mentioned here. The review by Bonner (*1*) of recent progress in studies with accessory growth substances should also be consulted.

A tentative classification of carbon compounds on the basis of nutritive properties has been proposed in a preliminary report (*20*) of some of the results on organic nutrition presented here. The groups suggested were calogens (energy) and formogens (molecular configuration). The latter included protosins (structural), biosins (chemical), and hormones (regulatory). Carbon compounds were considered to possess these properties in varying degree and to be capable of altering and particularly of supplementing their mutual deficiencies. The results since obtained are also readily interpreted on the basis of this grouping as phenomena of malnutrition and are practically identical in import with the experimental data on accessory growth factors.

In the absence of definite standards for comparison, the relative nutritiveness of carbon compounds can only be stated in general terms. Sucrose, *d*-glucose, *d*-fructose, and *d*-mannose are usually found to be excellent sources of carbon; glycerol and *d*-mannitol are rated as fair; and *d*-galactose and *d*-lactose are generally found to be poor. Data on *l*-sorbose are lacking. Lactose on hydrolysis forms

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[2] Italic numbers in parentheses refer to Literature Cited, p. 762.

an equimolecular mixture of glucose and galactose, but Pottevin (*9*) found *Aspergillus niger* unable to utilize it for growth, though it sufficed for respiration with the mature felt. Failure to grow on lactose was attributed to nonformation of lactase in the immature felts. Its presence in the mature felts was ascertained by Pottevin.

Previous studies by the writer (*15*, *20*) with the trace elements would indicate that the strain of *Aspergillus niger* employed requires no accessory growth factors and readily attains maximum normal growth with sucrose and inorganic nitrogen. Nielsen and Hartelius (*6*), and Nielsen and Sing-Fang (*8*) report the existence of another strain of this fungus requiring pyruvic and glycolic acids, however. Similar claims advanced by other investigators also would indicate the existence of variations in accessory requirements in the *Aspergillus niger* group similar to those existing among the yeasts. Nevertheless, the possibility that experimental accessory deficiency may in some cases be due to faulty carbon and nitrogen nutrition (*20*) and is not inherent in the organism has by no means been disproved.

All degrees of specificity in amino acid requirements for growth have been found to exist among the fungi. Papers by Ezekiel et al. (*2*), Nielsen and Hartelius (*7*), Leonian and Lilly (*5*), and others deal with this question. It is considered by Nielsen and Hartelius that β-alanine, *d*-lysine, *d*-arginine, and *d*-glutamic acid function with yeasts as accessory substances and not as sources of nitrogen. This would make a total of nine accessories required for growth of yeast, the others being *i*-inositol, aspartic acid, thiamin, pantothenic acid, and biotin (*4*). The writer (*20*) has pointed out the beneficial action of sodium iron chlorophyllin in conjunction with *i*-inositol on the growth of yeast; and with amino acids on the growth of *Aspergillus niger* with *d*-mannitol, *d*-lactose, and glycerol.

The mutual interdependence in composition of carbon and nitrogen compounds with respect to nutritiveness is not confined in all probability to determination of the supplementary amino acids and accessories required for growth by plants. Stirn and Arnold (*21*) were able to cure functional disturbances in rats due to thiamin deficiency by partial substitution of triacetin or tricaproin for carbohydrate. Of equal significance, perhaps, is the decrease in utilization of ingested lactose by the rat upon removal of butterfat from milk, reported by Schantz et al. (*13*). Whether other fungi found to require an external supply of thiamin, pyrimidine, or thiazole with a glucose-asparagine solution, as in the case of *Phycomyces* (*10*, *11*, *12*), will react similarly under all conditions remains to be determined. Thiamin deficiency in fungi may in some instances be associated with a high carbohydrate diet because of the presumable need for a greater quantity of thiamin than can be supplied at the rate at which the organism is capable of synthesizing it.

METHODS

The "W" strain of *Aspergillus niger* was grown on 50 cc. of a 5-percent sucrose solution in 200-cc. pyrex Erlenmeyer flasks for 4 days at 35° C. Ammonium nitrate, monopotassium phosphate, and magnesium sulfate ($7H_2O$) were also added to the nutrient solution in amounts of 2.06, 0.55, and 0.25 gm. per liter respectively. All chemicals were of reagent grade or the purest obtainable. The water

was redistilled in a pyrex glass still. Sterilization of media was effected by heating for 20 minutes in the steamer, except for cultures containing yeast extract. The latter were treated in the autoclave for 20 minutes at 15 pounds' pressure. The flasks were inoculated with a suspension of spores. The cultures were filtered with fritted glass crucibles of No. 3 porosity, and were dried overnight at 103° before weighing.

The organic substances tested for supplementary growth action included those listed in a previous paper (*17*) together with pimelic acid, nicotinic acid, androsterone, *d*-tocopherol, nutrose, yeast extract, malt extract, Witte peptone, Bacto-peptone, 3,5–*l*-diiodotyrosine, *dl*-threonine, and citric, glyceric, gluconic, glyoxylic, kojic, lactic, mucic, oxalic, and tartaric acids. Though 82 compounds were used, many known to be important in metabolism were unavailable for test. What other substances may be important in metabolism is of course as yet unknown. Another handicap consisted in the inability to obtain and test the decomposition products of the substances found to have a supplementary growth effect.

The method followed in the study of these organic substances was to determine which substance was the most effective and then test the effectiveness of the other substances when used in conjunction with it. This procedure was continued until growth was approximately maximum. Concentration of the individual supplementary substances was arbitrarily limited to 20 mg. per liter, i.e., 20 parts per million. This scheme was followed in its entirety, however, only with glycerol, and included thorough tests with a total of 77 chemical elements. In other cases the tests with the complete list of chemical elements were omitted, and, where an organic compound had been found capable of bringing about satisfactory growth, tests with the other organic substances were omitted as well.

Reference is made to purification experiments with glycerol. These were performed in a manner similar to that used with sucrose (*14*). A gram of calcium carbonate was added to the nutrient solution containing a slight excess of constituents, which was then heated and filtered while hot through a fritted glass filter of No. 4 porosity. This method was not employed with the other sources of carbon in the present series of experiments.

GROWTH WITH GLYCEROL

The data in table 1 are typical of the responses of *Aspergillus niger* when grown on glycerol. It will be noted that the yields in the first three experiments without scandium are 107.4, 57.5, and 21.7 mg. per culture, respectively, when the nutrient solution contained reagent glycerol, redistilled glycerol, or redistilled glycerol in a purified solution. Appreciable increases in yield were brought about through the addition of traces of scandium to the nutrient solution. These increases in growth could not be obtained with any of the other 76 chemical elements at the concentration levels tested. Moreover it would appear that the organism requires iron, zinc, copper, manganese, molybdenum, and gallium with glycerol as well as with sucrose. The poor results with trace-element deficiencies are considered to be due largely to the poor yields obtained relative to the level of trace-element impurity.

TABLE 1.—*Effects of a deficiency of trace elements on the growth of Aspergillus niger for 4 days at 35° C. with glycerol and with glycerol to which traces of organic nitrogen compounds had been added* [1]

Element omitted	Glycerol											
	Reagent				Distilled				Distilled and purified with $CaCO_3$			
	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	102.7	38.12	2.03	2, bl	36.3	66.48	.....	2, bl	4.3	7.43	.....	2, bl
Zn	231.4	85.89	1.88	4, bl	17.3	31.68	.....	2, bl	7.7	13.30	.....	2, bl
Cu	305.3	113.33	1.72	2, bl	40.3	73.81	.....	2, bl	53.0	91.54	.....	2, bl
Mn	303.4	112.62	1.84	0	49.6	90.84	.....	2, bl	196.4	339.20	.....	2, bl
Mo	243.7	90.46	1.77	4, bl	53.5	97.98	.....	2, bl	55.1	95.16	.....	2, bl
Ga	241.2	89.53	1.87	2, bl	101.5	185.90	.....	2, bl	25.9	44.73	.....	2, bl
Sc	107.4	39.87	2.01	2, bl	57.5	105.31	.....	2, bl	21.7	37.48	.....	2, bl
None	269.4	100.00	1.82	4, bl	54.6	100.00	.....	2, bl	57.9	100.00	.....	2, bl
Max.[3]	387.6	.....	.....	.....	104.4	.....	.....	.....	196.4	.....	.....	.....
C. U.[4]	.....	15.50	.....	.....	.....	4.18	.....	.....	.....	7.86	.....	.....
pH [5]	.....	.....	5.30	.....	.....	.....	4.75	.....	.....	.....	6.33	.....

Element omitted	Glycerol with indicated nitrogen compound (20 mg. per liter)											
	Witte peptone				Bacto peptone				Sodium iron chlorophyllin			
Fe	98.6	22.09	2.28	1, bl	72.0	21.44	2.33	2, bl	553.1	77.10	1.99	8, bl
Zn	455.3	102.02	1.89	1, bl	359.7	107.12	1.81	2, bl	301.5	42.03	1.91	10, bl
Cu	423.3	94.85	2.19	1, bl	448.5	133.57	2.28	2, bl	410.9	57.28	1.87	6, bl
Mn	903.1	202.35	3.79	1, bl	761.3	226.71	3.17	2, bl	422.6	58.91	1.77	8, bl
Mo	350.9	78.63	1.87	1, bl	280.1	83.41	1.87	2, bl	590.0	82.24	1.97	6, bl
Ga	446.1	99.95	2.18	1, bl	387.8	115.49	2.02	2, bl	684.1	95.36	2.29	6, bl
Sc	455.2	102.00	2.21	1, bl	334.4	99.58	1.96	2, bl	691.0	96.31	2.31	6, bl
None	446.3	100.00	2.28	1, bl	335.8	100.00	1.99	2, bl	717.4	100.00	2.36	6, bl
Max.[3]	903.1	.....	.....	.....	761.3	.....	.....	.....	744.3	.....	.....	.....
C. U.[4]	.....	36.12	.....	.....	.....	30.45	.....	.....	.....	29.77	.....	.....
pH [5]	.....	.....	4.67	.....	.....	.....	4.76	.....	.....	.....	5.01	.....

Element omitted	Glycerol with indicated nitrogen compound (20 mg. per liter)—Continued											
	l-Proline				*d*-Lysine				Hemoglobin			
Fe	86.5	17.83	2.39	8, bl	78.6	18.37	2.41	8, bl	227.2	41.56	1.93	8, bl
Zn	174.4	35.94	2.05	10, bl	118.6	27.72	2.22	8, bl	289.9	53.04	1.89	8, bl
Cu	501.7	103.40	2.02	6, bl	217.2	50.77	1.90	6, bl	543.4	99.41	2.09	6, bl
Mn	253.8	52.30	1.86	6, bl	173.4	40.53	1.99	6, bl	451.8	82.65	1.87	6, bl
Mo	416.4	85.82	1.83	6, bl	356.5	83.33	1.80	6, bl	522.2	95.53	1.97	6, bl
Ga	617.3	127.23	2.26	6, bl	456.4	106.69	1.91	6, bl	596.7	109.16	2.17	7, bl
Sc	552.4	113.85	2.16	6, bl	456.4	106.69	1.87	6, bl	582.3	106.53	2.09	7, bl
None	485.2	100.00	2.07	6, bl	427.8	100.00	1.92	6, bl	546.6	100.00	2.10	6, bl
Max.[3]	617.3	.....	.....	.....	456.4	.....	.....	.....	619.3	.....	.....	.....
C. U.[4]	.....	24.69	.....	.....	.....	18.26	.....	.....	.....	24.77	.....	.....
pH [5]	.....	.....	4.60	.....	.....	.....	4.49	.....	.....	.....	4.80	.....

[1] Each liter of nutrient solution contained the following ingredients: Carbon compound, 50.0 gm.; NH_4NO_3, 2.06 gm.; KH_2PO_4, 0.55 gm.; $MgSO_4.7H_2O$, 0.25 gm.; iron, 0.30 mg.; zinc, 0.20 mg.; copper, 0.05 mg.; manganese, 0.025 mg.; molybdenum, 0.02 mg.; gallium, 0.02 mg.; and scandium, 0.04 gm.

[2] Sporulation was estimated on a scale ranging from 0 (= sterility) to 10 (= maximum sporulation); color of spores is indicated in all experiments by abbreviations of the words white, yellow, tan, brown, and black.

[3] Maximum individual yield.

[4] Coefficient of utilization, or yield per 100 gm. of sucrose.

[5] Initial acidity of the nutrient solution.

An attempt to obtain improved results with the trace elements was made through the addition of supplementary sources of nitrogen displaying marked growth-promoting properties. These substances were selected on the basis of the comparative tests with organic compounds mentioned in the description of experimental procedures. The results of special tests with the most effective of these compounds are also given in table 1. Witte peptone proved most effective in supplementing the molecular deficiencies of glycerol. Bacto-peptone and sodium iron chlorophyllin were somewhat less effective. Lysine was least effective of the six organic nitrogen materials specially tested. Results with trace elements showed no improvement despite the appreciable increases in growth brought about through the addition of supplementary sources of nitrogen to the nutrient solution containing glycerol. The assumption is that the increase in mineral impurities brought about through their addition to the nutrient solution more than compensated for the beneficial action of increased yields for demonstrating trace-element deficiencies.

The specific nature of the molecular deficiencies with glycerol is not disclosed by the materials most effective in promoting growth. Particularly is this the case with Witte peptone and Bacto-peptone, both of which comprise an unknown mixture of protein-decomposition products, accessory factors, and inorganic salts (*17*). Sodium iron chlorophyllin, proline, and hemoglobin, however, are characterized by the presence of the pyrrole ring in their molecules. This ring or its tetrahydro derivative (pyrrolidine) is also without doubt present in Witte peptone and Bacto-peptone. Hammett (*3*) has claimed that the pyrrolidone group plays a special part in differentiation of the cell. Sodium magnesium chlorophyllin was not quite so effective in increasing the yield as was the iron salt. Miscellaneous tests with yeast (*Saccharomyces ellipsoideus*, A. T. C. C. No. 4097) indicated the magnesium salt to be more effective than the iron salt, especially in conjunction with *i*-inositol. Yield of yeast under these conditions was increased from 0.3 mg. to 4.8 mg. after 4 days' growth at 25° C. This compares favorably with a maximum yield of 18.4 mg. per culture of 50 ml. in a 5-percent sucrose solution containing 260 mg. of yeast extract per liter.

Similar tests of the growth-promoting properties of carbon compounds not containing nitrogen were also made and are given in table 2. These materials were relatively ineffective in comparison with the nitrogen compounds and were therefore supplied at a concentration of 1,000 mg. per liter instead of 20 mg. per liter. Sucrose seemed to be slightly more effective in promoting growth.

Sucrose was tested also at a concentration of 20,000 mg. per liter in order to demonstrate that the quantities of trace elements supplied to the fungus were ample for maximum growth with glycerol. This was considered advisable, since the quantities added had been determined to be the necessary minima with sucrose. Moreover, the possibility existed that glycerol might render the trace elements unavailable through formation of complex ions in which they were present in un-ionized form. This did not prove to be the case, however, since yield was maximum. The fact that yield did not exceed maximum in experiment 2 is due, of course, to the presence of only sufficient quantities of mineral constituents to give maximum yield.

TABLE 2.—*Effects of a deficiency of trace elements on the growth of Aspergillus niger for 4 days at 35° C. with glycerol supplemented with other carbon compounds* [1]

Element omitted	Glycerol with indicated carbon compound (grams per liter)											
	Sucrose, 1 gm.				Sucrose, 20 gm.				Citric acid, 1 gm.			
	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	134.3	17.97	2.61	6, bl	194.5	18.85	2.12	4, bl	75.6	17.15	2.41	6, bl
Zn	350.4	46.88	1.80	8, bl	561.6	54.43	1.79	10, bl	146.1	33.13	2.17	6, bl
Cu	789.5	105.63	2.26	6, t	1,213.9	117.64	3.07	10, t	245.1	55.57	1.94	6, bl
Mn	726.5	97.20	2.06	4, bl	1,182.0	114.55	2.26	1, bl	339.9	77.07	1.91	2, bl
Mo	718.4	96.12	2.08	8, bl	1,046.5	101.41	3.65	10, bl	435.8	98.81	1.86	8, bl
Ga	749.2	100.24	2.22	8, bl	1,036.1	100.41	3.92	10, bl	607.8	137.82	2.17	8, bl
Sc	768.6	102.83	2.23	8, bl	1,032.1	100.02	3.96	10, bl	244.2	55.37	2.03	6, bl
None	747.4	100.00	2.18	8, bl	1,031.9	100.00	3.81	10, bl	441.0	100.00	2.01	6, bl
Max. [3]	823.1				1,213.9				651.0			
C. U. [4]		32.92				48.56				26.04		
pH [5]			4.58				4.70				2.90	

Element omitted	Glycerol with indicated carbon compound (grams per liter)—Continued											
	Glycolic acid, 1 gm.				Pyruvic acid, 1 gm.				Glyceric acid, 1 gm.			
Fe	141.8	55.23	2.03	1, bl	70.1	22.06	2.21	2, bl	315.8	93.24	1.86	6, bl
Zn	246.8	96.14	1.95	2, bl	287.2	90.40	1.81	2, bl	109.0	32.18	2.04	10, bl
Cu	216.1	84.18	1.96	2, bl	257.4	81.02	1.84	2, bl	329.7	97.34	1.85	6, bl
Mn	702.3	273.59	3.24	0	603.2	189.86	2.36	0	676.1	199.62	2.38	4, bl
Mo	221.0	86.09	1.76	2, bl	257.4	81.02	1.83	2, bl	364.8	107.71	1.83	6, bl
Ga	233.2	90.84	1.92	2, bl	577.8	181.86	2.77	2, bl	368.8	114.20	1.86	6, bl
Sc	267.0	104.02	1.92	3, bl	345.7	108.81	1.96	2, bl	206.7	61.03	2.06	6, bl
None	256.7	100.00	2.03	2, bl	317.7	100.00	1.92	2, bl	338.7	100.00	1.86	6, bl
Max. [3]	702.3				603.2				711.7			
C. U. [4]		28.09				24.13				28.47		
pH [5]			2.99				2.55				3.07	

[1] See footnote 1, table 1.
[2] See footnote 2, table 1.
[3] See footnote 3, table 1.
[4] See footnote 4, table 1.
[5] See footnote 5, table 1.

Attention is also directed to the results of deficiency tests with scandium in solution supplemented with citric acid and glyceric acid, giving yields of 55.35 and 61.05 percent of maximum respectively. The reason for these responses is unknown but it might be associated with the fact that these acids may be products of metabolism of this fungus. Results of deficiency tests with other elements and in other solutions were uniformly poorer than those usually obtained with sucrose. The sucrose, however, was of an especially high standard of purity (0.0014 percent ash).

GROWTH WITH SOURCES OF CARBON OTHER THAN GLYCEROL

Tests of trace-element requirements were also extended to include glucose, fructose, mannose, galactose, sorbose,[3] mannitol, and lactose. The data for all but the last two are tabulated in table 3. Mannitol and lactose have been omitted because practically no growth of the

[3] The sorbose used was obtained through the courtesy of the Bureau of Chemistry and Soils, U. S. Department of Agriculture. It had been produced through the fermentation of *d*-sorbitol by *Bacterium xylinum* and was considered better than 99 percent pure.

TABLE 3.—*Effects of a deficiency of trace elements on the growth of Aspergillus niger for 4 days at 35° C. with different carbon compounds*[1]

Element omitted	Sucrose				*d*-Glucose				*d*-Fructose				*d*-Mannose			
	Yield per 2.5 gm. sucrose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. glucose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. fructose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. mannose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	57.3	6.54	2.46	2, bl	478.3	43.31	1.80	4, bl	1,009.0	95.57	1.95	7, bl	707.3	77.93	2.53	2, bl
Zn	32.9	3.75	2.97	2, bl	73.9	6.69	2.57	6, bl	90.9	8.61	2.49	10, bl	24.4	2.69	3.28	4, bl
Cu	688.2	78.52	2.41	1, bl	1,111.1	100.61	2.90	4, br	1,009.6	95.63	2.65	6, br	805.5	88.75	3.05	2, bl
Mn	791.9	90.35	2.11	2, bl	1,101.3	99.72	1.94	2, bl	1,045.3	99.01	2.34	10, bl	1,031.7	113.67	3.44	4, bl
Mo	440.4	50.25	1.74	2, bl	806.1	72.99	1.92	4, bl	873.1	82.69	2.20	6, bl	463.5	51.07	1.89	2, bl
Ga	834.1	95.16	2.44	6, bl	1,150.6	104.18	2.56	8, bl	1,058.1	100.22	2.38	10, bl	836.6	92.17	3.16	2, bl
Sc	838.4	95.65	2.48	6, bl	1,148.3	103.97	2.80	8, bl	1,053.2	99.75	2.54	10, bl	878.7	96.81	3.50	2, bl
None	876.5	100.00	2.46	6, bl	1,104.4	100.00	2.76	10, bl	1,055.8	100.00	2.44	10, bl	907.6	100.00	3.02	2, bl
Max.[3]	957.7				1,150.6				1,076.3				1,031.7			
C. U.[4]		38.31				46.02				43.05				41.27		
pH[5]			6.91				7.15				7.15				6.76	

Element omitted	*d*-Galactose[1]				*l*-Sorbose				*l*-Sorbose[1]			
	Yield per 2.5 gm. galactose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. sorbose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]	Yield per 2.5 gm. sorbose	Proportion of maximum yield	Acidity at harvest	Sporulation[2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	60.8	101.84	2.55	0	730.2	93.27	3.15	2, bl	903.8	92.86	4.23	2, bl
Zn	60.6	101.51	2.54	0	594.9	75.98	2.27	2, bl	923.2	94.86	3.88	1, bl
Cu	58.3	97.65	2.55	0	762.1	97.34	2.76	2, bl	958.3	98.46	4.25	6, bl
Mn	56.6	94.81	2.53	0	860.1	109.86	3.70	2, bl	978.2	100.51	4.29	0
Mo	58.3	97.65	2.52	0	512.9	65.51	2.19	2, bl	497.6	51.12	2.35	1, bl
Ga	61.0	102.17	2.53	0	788.5	100.72	3.14	2, bl	958.5	98.48	3.86	6, bl
Sc	59.5	99.66	2.55	0	808.1	103.22	3.12	2, bl	947.8	97.38	4.35	6, bl
None	59.7	100.00	2.52	0	782.9	100.00	2.87	2, bl	973.3	100.00	4.15	6, bl
Max.[3]	61.0				860.1				978.2			
C. U.[4]		2.44				34.40				39.13		
pH[5]			3.93				6.82				4.68	

[1] See footnote 1, table 1. K_2HPO_4, 0.35 gm. was substituted for KH_2PO_4, 0.55 gm., in all but the fifth and seventh experiments.
[2] See footnote 2, table 1.
[3] See footnote 3, table 1.
[4] See footnote 4, table 1.
[5] See footnote 5, table 1.

fungus occurred with these sources of carbon. Little doubt exists that the trace elements determined to be necessary with sucrose are also necessary with these other sources of carbon. Their degree of freedom from trace-element impurities was not sufficiently great to disclose whether special requirements do or do not exist. Repetition of these experiments at a higher level of purity would, therefore, be advisable.

Sucrose, glucose, fructose, mannose, and sorbose seemed equally effective for the nutrition of *Aspergillus niger*. Galactose was quite inadequate, however. The results with *l*-sorbose may need revision, since the responses with this source of carbon varied somewhat more in successive experiments than did those with the other carbon compounds.

The supplementary effect of traces of organic nitrogen compounds on growth with glycerol (table 1) appeared to offer a possible explanation for the relative unavailability of lactose, mannitol, and galactose, as well as glycerol. This interpretation was that substitutions of a poor carbon source for sucrose resulted in the induction of accessory-substance and amino acid requirements for supplementing the deficiency in necessary molecular configurations of the poor carbon source. The tests with organic supplements for glycerol were therefore continued further. The first two experiments of table 4 show the final results of these tests.

In the first experiment of table 4, the addition of 20 mg. per liter each of sodium iron chlorophyllin and β-phenylalanine increased the yield with glycerol to 906.1 mg., and the further addition of *l*-tyrosine increased the maximum yield to 961.6 mg. in experiment 2. That is to say, a total of 1 mg. each of these supplementary or accessory factors per culture sufficed to increase the yield from a maximum of 269.4 mg. (experiment 1, table 1) to one of 961.6 mg. It is logical to assume that continued tests for effective supplements would have resulted in a yield fully equal to that obtainable with sucrose, etc. Further investigations will, it is believed, lead to the discovery of more effective supplementary factors.

The results of similar studies with lactose are given in the third experiment of table 4. Sodium iron chlorophyllin again proved most effective, and sucrose was next in promoting growth. The other organic compounds tested proved ineffective, presumably because of the limited number available for trial. Results with galactose were even poorer at the supplementary level (20 mg. per liter) used. The poor results with lactose and galactose are attributed wholly to lack of addition of the appropriate compound to supplement the molecular deficiencies of these two compounds. This is not surprising, since less than 100 compounds were available for trial. The data in table 5 afford convincing proof that lactose and galactose are assimilable by *Aspergillus*, under appropriate conditions, also.

The last two experiments of table 4 illustrate the results obtained with mannitol when supplemented with traces of organic nitrogen. Sodium iron chlorophyllin again proved most effective, followed by *d*-glutamic acid, and *dl*-α-alanine. The action of these substances when the organism is grown on mannitol cannot be regarded as evidence for the requirement of accessory growth factors. Maximum yields are reached in 7 days by *Aspergillus* without their addition (*19*).

TABLE 4.—*Effects of a deficiency of trace elements on the growth of Aspergillus niger for 4 days at 35° C. with glycerol, lactose, and mannitol in the presence of supplementary compounds of organic nitrogen* [1]

Element omitted	Glycerol with 20 mg. per liter of sodium iron chlorophyllin and β-phenylalanine				Glycerol with 20 mg. per liter of sodium iron chlorophyllin, β-phenylalanine, and *l*-tyrosine				Lactose with 20 mg. per liter of sodium iron chlorophyllin and 1.0 gm. per liter of sucrose				Mannitol with 20 mg. per liter of sodium iron chlorophyllin and *d*-glutamic acid				Mannitol with 20 mg. per liter of sodium iron chlorophyllin, *d*-glutamic acid, and *dl*-α-alanine			
	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. glycerol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. lactose	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. mannitol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. mannitol	Proportion of maximum yield	Acidity at harvest	Sporulation [2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	791.4	93.81	2.33	6, t	814.2	85.81	2.59	4, br	284.1	95.37	1.82	10, bl	662.1	84.69	2.40	6, bl	718.5	84.33	2.83	4, bl
Zn	478.2	56.69	1.76	8, br	532.1	56.08	1.78	8, bl	223.4	75.00	1.82	10, bl	105.1	13.44	2.43	8, bl	156.4	18.36	2.31	8, bl
Cu	635.3	75.31	2.18	4, y	634.5	66.87	2.20	2, br	289.4	97.15	1.79	10, bl	365.9	46.80	2.07	4, bl	302.2	35.46	1.83	4, bl
Mn	458.6	54.37	1.78	6, bl	499.7	52.66	1.83	4, bl	298.3	100.14	1.78	10, bl	265.7	33.99	1.86	8, bl	374.1	43.90	1.85	8, bl
Mo	588.4	69.74	1.75	8, t	637.2	67.15	1.83	4, br	295.6	99.22	1.79	10, bl	473.9	60.62	1.93	6, bl	482.1	56.58	1.93	6, bl
Ga	841.7	99.77	2.93	6, t	911.9	96.11	3.16	4, br	286.3	96.11	1.79	10, bl	614.8	78.65	2.50	6, bl	742.8	87.18	3.29	6, bl
Sc	847.5	100.46	2.74	6, t	924.9	97.48	3.06	6, br	286.6	96.20	1.79	10, bl	796.0	101.82	2.98	6, bl	710.2	83.35	3.62	6, bl
None	843.6	100.00	2.45	8, t	948.8	100.00	3.34	6, br	297.9	100.00	1.79	10, bl	781.7	100.00	2.69	6, bl	852.1	100.00	3.38	6, bl
Max. [3]	906.1				961.6				301.6				796.0				935.6			
C. U. [4]		36.24				38.46				12.06				31.84				37.42		
pH [5]			5.08				3.86				3.93				4.62				4.62	

[1] See footnote 1, table 1.
[2] See footnote 2, table 1.
[3] See footnote 3, table 1.
[4] See footnote 4, table 1.
[5] See footnote 5, table 1.

This would appear conclusive evidence that the fungus is entirely capable of their synthesis, though not perhaps at a rate as high as that at which they can be utilized.

The effects of trace-element deficiencies in the experiments with supplementary compounds were much the same as in those previously discussed. It appears probable that iron, zinc, copper, manganese, molybdenum, and gallium are required under all conditions.

EFFECT OF ADMIXTURE OF CARBON COMPOUNDS ON ASSIMILABILITY

That the effect of chlorophyllin and amino acids is actually to supplement deficiencies in molecular configuration is rendered clearer by the results given in table 5. These show the yields obtained with mannitol, glycerol, lactose, and galactose both singly and in mixture. All four of these poor carbon sources proved available for growth on proper admixture. The results with mannitol and galactose proved exceptionally interesting in view of the failure to find a suitable accessory for the latter in the previous experiments. Though the yield with mannitol was only 34.3 mg. and that with galactose 27.9 mg., the yield with a mixture of these compounds was 392.6 mg. Supplementation of mannitol with lactose also improved assimilation. It would be interesting to learn the relative assimilability of these sources in mixtures of even greater complexity and in the presence of supplementary nitrogen compounds.

TABLE 5.—*Effect of equal admixtures (total of 2.5 gm. per culture) of poor carbon sources on yields of Aspergillus niger* [1]

Carbon compound	Compound used per culture	Yield	Mixture (2.5 gm. per culture)	Yield	
				Computed	Found
	Grams	*Mg.*		*Mg.*	*Mg.*
d-Mannitol	1.25	4.5	*d*-Mannitol+glycerol	231.2	545.1
Glycerol	1.25	226.7	*d*-Mannitol+*d*-lactose	21.4	233.6
d-Lactose	1.25	16.9	*d*-Mannitol+*d*-galactose	21.5	392.6
d-Galactose	1.25	17.0	Glycerol+*d*-lactose	243.6	458.3
d-Mannitol	2.5	34.3	Glycerol+*d*-galactose	243.7	154.7
Glycerol	2.5	349.0	*d*-Lactose+*d*-galactose	23.9	16.7
d-Lactose	2.5	74.6			
d-Galactose	2.5	27.9			

[1] See footnote 1, table 1, for quantities of mineral constituents employed.

Experimental data showing the effect of trace-element deficiencies on growth and development with mixtures of inadequate carbon sources have been tabulated in table 6. Omission of any one of the trace elements appears to result in a slight but definite decrease in yield. With compounds of higher purity the effects of these omissions would have been enhanced, it is believed. Nonaddition of scandium also brought about a decrease in yield of a magnitude too great to be attributed to normal experimental variations.

Though a continuation of these experiments with a nutrient solution purified with calcium carbonate would probably have led to greater differences with trace-element deficiency, this method was not employed. Neither were any experiments carried out to ascertain

the effects of admixture of more than two sources of carbon, nor of a combination of this procedure with tests of supplementation with various organic nitrogen compounds.

TABLE 6.—*Effect of deficiencies in trace elements on utilization of carbon compounds in admixture by Aspergillus niger grown for 4 days at 35° C.*[1]

Element omitted	Mixture of *d*-mannitol and *d*-galactose				Mixture of *d*-lactose and glycerol			
	Yield per 2.5 gm. of carbon sources	Proportion of maximum yield	Acidity at harvest	Sporulation [2]	Yield per 2.5 gm. of carbon sources	Proportion of maximum yield	Acidity at harvest	Sporulation [2]
	Milligrams	*Percent*	*pH*		*Milligrams*	*Percent*	*pH*	
Fe	301.7	80.51	1.74	0	398.2	69.90	1.88	2, bl
Zn	344.2	91.86	1.74	0	328.2	57.61	1.74	3, bl
Cu	353.4	94.31	1.87	0	497.6	87.36	2.03	8, bl
Mn	399.4	106.59	1.94	0	533.8	93.72	2.14	2, bl
Mo	247.1	65.94	1.80	0	515.4	90.49	2.10	6, bl
Ga	351.1	93.70	1.80	0	493.2	86.59	2.07	2, bl
Sc	316.4	84.44	1.80	0	508.7	89.30	2.09	2, bl
None	374.7	100.00	1.88	0	569.6	100.00	2.16	2, bl
Max.[3]	399.4				600.4			
C. U.[4]		15.98				24.02		
pH [5]			3.81				3.75	

[1] See footnote 1, table 1.
[2] See footnote 2, table 1.
[3] See footnote 3, table 1.
[4] See footnote 4, table 1.
[5] See footnote 5, table 1.

EFFECT OF AN EXTERNAL SUPPLY OF METABOLIC PRODUCTS

Growth of *Aspergillus niger* in the dibasic optimum solution with sucrose is equal to or greater than that of other hexoses, being approximately 1,150 mg. dry weight per culture of 50 cc. (2.5 gm. sucrose) after 4 days at 35° C. The average rate of growth, therefore, is almost exactly 12 mg. per hour, or 1.04 percent per hour of the maximum yield in a solution in which the organism is compelled to synthesize all its metabolic products from sucrose and inorganic nitrogen.

The experimental data in table 7 show the effect of the metabolites in yeast extract on the growth of *Aspergillus* when substituted in part for sucrose. The strain of *Aspergillus* employed in this experiment does not require the presence of accessory growth factors. The mineral constituents were present in optimum amount, only sucrose being decreased from 50 gm. per liter to 40 gm. In every case the yield with yeast extract was greater than that with an equivalent quantity of sucrose over and above the basic 40 gm. per liter. A yield of 1,214 mg. was reached in 3 days with 40 gm. of sucrose and 8 gm. of yeast extract per liter. The average rate of growth was 17 mg. per hour, or 1.4 percent per hour of maximum yield. The yield with 50 gm. of sucrose per liter had reached a value of only 1,043.4 mg. after 4 days of growth. The beneficial effects of yeast extract are especially striking during the early stages of growth. The average rate of growth was 11 mg. per hour, or 1.05 percent per hour of maximum yield.

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TABLE 7.—*Increased efficiency of carbon utilization by Aspergillus niger at 35° C. through partial replacement of sucrose by yeast extract* [1]

Growth period	Substance added	Yields obtained with indicated number of grams per liter of substance added to solution containing 40 gm. of sucrose per liter					
		0	2	4	6	8	10
Days		*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*
[2] 2	Sucrose		218.6	165.7	172.2	159.7	229.5
	Yeast extract		859.1	981.2	1,036.3	1,063.0	1,065.8
3	Sucrose	689.2	702.5	743.8	761.9	749.7	
	Yeast extract		1,083.1	1,125.6	1,172.8	1,214.0	1,224.3
4	Sucrose	894.6	856.2	941.7	997.4	1,048.4	1,043.4
	Yeast extract		971.3	1,021.7	1,083.0	1,103.5	1,140.5

[1] See footnote 1, table 3, except that sucrose was employed at 40 gm. per liter.
[2] Sporulation had not begun in the sucrose cultures, whereas the yeast cultures were black with spores.

DISCUSSION

Nothing requiring special comment is presented by the results on trace-element requirements of the fungus, except in the case of scandium. Scandium proved quite beneficial to growth of the organism when glycerol was supplied as a source of carbon. Its nonaddition with other sources of carbon was quite without effect, however, and differs in this respect from the results obtained with the other trace elements. Iron, zinc, copper, manganese, molybdenum, and gallium usually gave indications of their essentiality for metabolism with all sources of carbon, even though the differences were sometimes slight. This might have been anticipated in view of the lesser freedom from mineral impurities of these carbon compounds as compared with sucrose. A possible relation may exist, therefore, between the low molecular weight of glycerol, necessitating as it does a greater degree of metabolic molecular condensations, and the ability to demonstrate its action on the fungus. The specific action of scandium with glycerol was subjected to repeated tests with a large number of the chemical elements, and there would seem to be little doubt that it exists.

Growth with reagent glycerol in the absence of scandium amounted to only 107.4 mg., and decreased to 57.5 mg. when the glycerol was redistilled in a pyrex glass still. Purification with calcium carbonate of the nutrient solution containing resdistilled glycerol caused a further decrease in yield to 21.7 mg. The yields in the presence of scandium were 269.4 mg., 54.6 mg., and 57.9 mg. respectively. Addition of even highly purified sucrose to the medium in small quantity (1 gm. per liter) eliminated the effect of scandium. Other organic substances tried behaved similarly, except citric acid and glyceric acid.

Growth of the fungus proved optimum on sucrose, glucose, fructose, mannose, and sorbose. Yields with lactose, galactose, and mannitol were very poor. The effect of the time element on this response is known for mannitol (*19*) only, and indicates that maximum yield requires 7 days for its attainment as compared to 4 days with sucrose. It appears to differ in this respect from glycerol, lactose, and galactose.

The experimental data on the carbon nutrition of the organism obtained under these circumstances have proved to be of considerable interest. Briefly, they comprised evidence that poor sources of carbon (*d*-mannitol, *d*-galactose, *d*-lactose, glycerol) gave marked

increases in growth on admixture. It was found further that substitution of a poor carbon source for sucrose resulted in the induction of special amino acid and accessory-factor requirements.

The response of the organism when grown with glycerol or mannitol differed greatly from that with lactose and galactose upon the addition of small quantities of other organic compounds. The substances that were tested have been listed in a previous paper (*17*). Yields with glycerol were practically maximum in the presence of traces of sodium iron chlorophyllin, β-phenylalanine, and *l*-tyrosine. The same was true for mannitol in the presence of traces of sodium iron chlorophyllin, *d*-glutamic acid, and *dl*-α-alanine. A distinct though slight effect was produced by sodium iron chlorophyllin with lactose, though other nitrogen compounds proved ineffective. Traces of none of the compounds available for test proved effective in aiding the growth of the fungus on galactose.

The effects obtained by the addition of traces of organic nitrogen compounds to glycerol and mannitol are interpreted as due to the induction of special nitrogen requirements. Glycerol and mannitol are probably poor sources of carbon because they do not furnish the specific molecular groups required by the organism in the synthesis of organic nitrogen. This view is in agreement with the results obtained with carbon compounds in admixture.

Perhaps the most interesting feature encountered was the discovery that rate of growth with an optimum solution containing sucrose and inorganic nitrogen could be greatly increased by the addition of yeast extract. The acceleration in growth is presumed to be brought about by the presence in yeast extract of many of the metabolic products that *Aspergillus* usually has to synthesize from sucrose and inorganic nitrogen. That this is the correct interpretation is rendered more probable by the accompanying slight but definite increases in efficiency of carbon utilization with yeast extract. The identification and production in a high state of purity of the metabolic products responsible for the acceleration of growth would be highly desirable in connection with studies dealing with the functions of the trace elements.

SUMMARY

Trace-element and carbon-compound requirements were studied with cultures of *Aspergillus niger* Van Tiegh. grown for 4 days at 35° C. The results on trace-element needs with the sources of carbon used were poorer than those obtained with sucrose of exceptional purity.

Iron, zinc, copper, manganese, molybdenum, and gallium would appear to be necessary whatever the source of carbon. Scandium exhibited biological specificity with the fungus when grown on glycerol, the yield being doubled.

Sucrose, *d*-glucose, *d*-fructose, *d*-mannose, and *l*-sorbose proved to be excellent sources of carbon and equally effective for nutrition, whereas glycerol, *d*-mannitol, *d*-lactose, and *d*-galactose proved quite ineffective.

Admixture of these carbon compounds, incapable of assimilation when available as sole carbon sources, was found markedly to increase their assimilability. Increased assimilability on admixture was interpreted on the basis of mutual supplementation of compounds deficient in essential molecular configurations.

Growth with glycerol and mannitol could also reach maximum when these compounds were supplemented with traces of sodium iron chlorophyllin and specific amino acids. The beneficial effects of traces of supplementary nitrogen compounds on carbon sources of low assimilability is attributed to the induction of special requirements for supplementation of deficiencies in molecular configuration. They are not considered due to the necessity for accessory growth factors, since the organism is fully capable of maximum growth with inorganic nitrogen and sucrose.

Tests with yeast extract indicated that a supply of metabolites on a sucrose medium caused acceleration in growth, under otherwise optimum conditions, not attributable to accessory-factor requirements. These effects of organic nitrogen supplementation were due to the existence of limiting factors in functional processes of the fungus and not to absence of the function.

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AMINO ACIDS IN THE CORN KERNEL [1]

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INTRODUCTION

A knowledge of the amino acid composition of individual staple foods used in human and animal dietary is of value to the nutritional worker as well as to the agriculturist. The classification of amino acids as dispensable and indispensable emphasizes the protein quality rather than the quantity requirement of protein in the daily diet. We do not eat or feed purified proteins of known amino acid composition. From the nutritional standpoint, staple foods are generally consumed in combinations in order to correct or supplement certain deficiencies in amino acids, vitamins, minerals, etc., and thus avoid the use of concentrates. The present study in the series of amino acids in staple foods is a further attempt to furnish data on corn (*Zea mays* L.).

Pellagra has been found to occur in sections where the population is poorly nourished and lives chiefly on corn. This observation suggested the nutritional origin of the disease, and the low quality of corn proteins was suspected as a contributing factor to the malnourished state of pellagrins. Osborne and Clapp (*5*) [2] analyzed zein, the alcohol-soluble corn protein, and noted the absence of tryptophane and lysine, both of which are nutritionally indispensable amino acids. In the corn glutelin, the author has found 0.516 percent of tryptophane (*1*); thus the absence of tryptophane in the zein is largely, if not wholly, corrected when whole corn is eaten.

EXPERIMENTAL METHODS

The method for determining amino acids has been described in two previous publications (*2*, *3*). Certain changes have been introduced, which are given in the following paragraph, to suit the analytical procedure to the property of the protein material present in the corn kernel.

A white corn and a yellow corn [3] were selected for analysis. The cleaned and selected whole corn kernels were ground to a fine meal and stored at a temperature slightly below freezing. Duplicate samples (air-dry) of 25 gm. each were used for analysis. Ether extraction to remove fatty substances was omitted, because it lowers the solubility of protein in a 1-percent salt solution (*2*). The finely ground corn meal was, therefore, extracted with 100 cc. of precooled 1-percent NaCl solution. Three of these salt extractions of 1-hour duration at 6°–8° C. were applied for removal of the water- and salt-

[1] Received for publication May 15, 1939. This is the third in a series of papers on amino acids in staple foods.

[2] Italic numbers in parentheses refer to Literature Cited, p. 768.

[3] The author is indebted to M. T. Jenkins, of the Bureau of Plant Industry, and to H. C. Fellows, of the Bureau of Agricultural Economics, U. S. Department of Agriculture, for supplying and grinding the corn samples.

soluble proteins. Then the residue was extracted twice with 80-percent alcohol in 100-cc. portions at room temperature. One of these alcoholic extractions was of 3 hours' duration, and another lasted overnight. The third alcoholic extraction was carried out at 56°–58° C. for only one-half hour. The residue was cooled in a refrigerator, and the starch removed by the addition of a 21-percent HCl solution, using 200 cc. the first time at refrigeration temperature for 1 hour and stirring occasionally to facilitate the dispersion of starch. The acid extract separates well from residue "R" by centrigutation. By the addition of an equal volume of 95-percent alcohol to the acid extract the starch precipitates. The extract was then centrifuged and the supernatant liquid was decanted. The decanted liquid was then evaporated to a small volume and added to the 1-percent NaCl and 80-percent alcohol extracts. The residue "R" was extracted twice more with 21-percent HCl to remove all starch, 50 to 100 cc. of acid being used each time. These acid extracts do not separate clearly by centrifugation and the slightly turbid supernatant liquid has to be filtered, preferably in the refrigerator. Since the second and third acid extracts contained only starch and an insignificant quantity of nitrogen, the filtrate was discarded. The precipitate from the filtrate, however, was removed and added to the starch-freed residue and hydrolyzed for 24 hours in 20-percent HCl. This hydrolysate, together with the hydrolysate of the combined salt, alcohol, and acid-alcohol extracts, was analyzed for the amino acids as described in previous publications (*2*, *3*).

The distribution of nitrogen in the extracts already referred to was approximately the same in the white and yellow corn samples and together represented from 90 to 92 percent of the total nitrogen.

The salt- and alcohol-insoluble type of protein constituted the largest percentage (48 percent); the 80-percent alcohol-soluble protein, 27 percent; and the 1-percent NaCl solution, 16 percent. It should be noted that the distribution of nitrogen in the different extracts is comparable only when the several factors, such as fineness of ground particles of meal, the concentration of salt or alcoholic solution, the order of the extraction by the different solvents, and the varieties under investigation, are identical. An 80-percent alcoholic solution, for example, invariably removed more nitrogen than an 85-percent solution in the procedure just described.

In regard to the determinations of tryptophane [4] and histidine, a few remarks are needed. By using a Bürker colorimeter, which is equipped with color compensation chambers, a decided improvement was observed in color matching by the May and Rose tryptophane method as modified by the author (*1*). The Ehrlich reagent was omitted from the samples used for color compensation, otherwise the technique was similar to that used for the standard and for the unknown samples. The color compensation for both standard and unknown solutions results in a more accurate color match. In regard to the histidine determination, it was found advantageous to decolorize the solution with Carboraffin before it was precipitated by Hopkin's mercuric sulfate reagent.

[4] An error in calculation was discovered in the tryptophane percentages in the earlier papers (*3*, *6*). To obtain correct values those given for wheat, bran, and shorts should be doubled.

EXPERIMENTAL DATA

Showalter and Carr (7) state that the proportion between zein and the other proteins varies according to the nitrogen content of the corn.

The nitrogen content of the two types of corn selected for this investigation differed little, as is shown in table 1; therefore a difference found in the amino acid composition might be significant in selecting one or the other on the basis of protein quality. The analytical results as shown in table 1, however, do not reveal any justification for preferring white corn to yellow corn with respect to amino acid composition. On a dietary regimen where the protein requirement is covered only by the corn proteins unsatisfactory growth of animals has been reported (4), not because of lack of any specific amino acid, but rather because of insufficient quantities of some of those indispensable ones considered herein. This conclusion is reached on the basis of Rose's figures representing the minimum quantities of indispensable amino acids required to support growth (6). Furthermore, in table 2, where the amino acids are expressed in quantities as obtained from 1 gm. of nitrogen of the whole corn kernel, the inferior quality of the corn protein as compared to casein is clearly demonstrated in respect to tryptophane and lysine. A judiciously selected mixed diet, however, should correct a poor nutritional state brought about by a diet in which corn is the chief source of protein.

TABLE 1.—*Amino acid content and total nitrogen of two varieties of moisture-free corn grown in 1937*

Place grown and variety of corn	Cystine	Trypto-phane	Tyrosine	Arginine	Histi-dine	Lysine	Total nitrogen
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Virginia: Boone County White	0.096	0.047	0.703	0.212	0.089	0.107	1.71
Iowa: Black Yellow Dent	.095	.053	.700	.226	.109	.108	1.78

TABLE 2.—*Protein quality of indicated amino acid obtained from 1 gm. of staple food nitrogen with casein for comparison*

Staple food	Cystine	Trypto-phane	Tyrosine	Arginine	Histidine	Lysine
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
White corn	56	28	441	124	52	63
Yellow corn	53	30	393	127	61	61
Casein	20	130	405	236	156	475

SUMMARY

The present study shows definitely that none of the indispensable amino acids considered herein are missing from the whole corn flour and that they are equally distributed in white and yellow corn. The amino acid composition of the whole corn kernel as found and as described in this paper when compared with that of casein shows that tryptophane and lysine are present at a lower level. The deficiency of tryptophane and lysine, which are absent in zein, apparently is not corrected sufficiently by the rest of the corn proteins. This conclusion, based on analytical findings, supports the general feeding practice of supplementation.

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EXPERIMENTAL TRANSMISSION OF BOVINE VENEREAL TRICHOMONIASIS[1]

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INTRODUCTION

In spite of the fact that knowledge concerning bovine venereal trichomoniasis has been obtained only during the last 10 years, it does not follow that the disease is new. Probably it has existed for a long time. A review of the literature and of the salient facts concerning the disease was given in a previous paper by Rees.[2] That review showed that much of the work of previous investigators was epizoological and that there is need of long-term experiments to supplement existing data.

The specific objectives of the experiments recorded in this paper were as follows: (1) To observe the effects, on the fecundity of cows, of venereal trichomoniasis transmitted either by intravaginal inoculation of the parasite *Trichomonas foetus*, from bacteria-free and virus-free cultures, or by coitus with infected bulls; (2) to determine whether these female hosts would recover, and, in the event of recovery, whether they would be susceptible to reinfection; and (3) to obtain data on infection in the genital tract of the bull.

MATERIALS AND METHODS

In the experiments reported in this paper 10 heifers and 3 bulls were used. All were free from *Brucella abortus* infection and from tuberculosis, and at the beginning, from trichomoniasis also. Seven of the heifers, after an abortion or the birth of a calf, were again used for the experimental transmission of the disease. These animals were kept in ½-acre paddocks provided with adequate shelter and had access to a limited quantity of green feed and an ample quantity of hay, grain, and minerals.

The parasites (*Trichomonas foetus*) used for inoculating the females were freed from bacteria either by the method of Glaser and Coria [3] or of Rees [4] and cultivated in modified egg-Ringer-serum medium of Boeck and Drbohlav.[5] Infection of the females was accomplished either by intravaginal inoculation of *T. foetus* or through coitus with an infected bull. Inoculation was performed with a glass cannula to which a rubber bulb was attached. About 15 cc. of Ringer's-serum fluid in which the trichomonads were growing was inoculated high up in the vagina near the os uteri. The infected bull used in the transmission experiments acquired the infection through coitus with a heifer that had been infected through inoculation. Regardless of the

[1] Received for publication July 11, 1939.

[2] REES, CHARLES W. BOVINE VENEREAL TRICHOMONIASIS. North Amer. Vet. 19 (4): 33-36, illus. 1938.

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[5] BOECK, WILLIAM C., and DRBOHLAV, JAROSLAV. THE CULTIVATION OF ENDAMOEBA HISTOLYTICA. Amer. Jour. Hyg. 5: 371-407, illus. 1925.

method used to infect the cattle, no bacteria or viruses were introduced into the animals.

To determine the onset and the duration of the infection the cattle were examined twice a week, beginning at the time of exposure. The method of examination was the same as that described previously by Rees.[6]

EXPERIMENTAL DATA

RESULTS WITH FEMALES

The experimental data on the females are recorded in table 1. The following significant points are shown: (1) Heifer 161 failed to calve and was positive for trichomonads for almost 2 years, during which time there were no heat periods. (2) Five other animals (Nos. 157, 158, 160, 163, and 174) when exposed as heifers, and Nos. 155 and 156 when exposed as cows, all became positive for *Trichomonas foetus*, some of them remaining positive for as long as 136 days. While positive these animals either had irregular heat periods or failed entirely to show signs of oestrum; no conceptions occurred. (3) Heifer 159 was a nonbreeder. (4) Heifer 162, the only infected heifer showing no delay in calving, became pregnant first and was exposed to the disease later; in this case the cervical seal appears to have kept the trichomonads out of the uterus. As a cow, this animal showed little resistance. (5) Four animals, Nos. 157, 158, 160, and 163, that had been infected as heifers and had lost the infection, became reinfected through coitus but were positive for only 1 to 22 days; they became pregnant without appreciable delay.

Table 1 shows (1) that more than half of all examinations were positive, the percentage being 58; (2) that some samples appearing negative on immediate examination were positive after incubation; and (3) that some samples positive on immediate examination were negative after incubation. It is evident, therefore, that a combination of both examinations yielded more positives than would have been detected if only one method had been relied upon. A fact not shown in the table is that in most of the cases there were periods when all examinations were positive, alternating with periods when most of them were negative. As a rule, there was a period of heat near the close of the period of positive examination and usually, when bred at that time, the animal became pregnant.

The following explanation is offered for the lack of data on the incubation period and the duration of the infection of heifer 163: This animal was intended as a control and was served on April 9, 1937, by bull 150 which was thought to be noninfected. The reason for regarding this bull as noninfected was that between August 20, 1936, and March 1, 1937, the animal had been examined 14 times and found to be negative for *Trichomonas foetus*. With several exceptions, the examinations were of material from three sources, namely, the seminal vesicles, the vasa deferentia, and the prepuce. Examinations were made at the time that the samples were obtained and also after incubation in vitro. However, the examinations made on June 23, 1937, 75 days after coitus of bull 150 with heifer 163, showed both the heifer and the bull to be positive for *T. foetus*. Since, prior to April 9, heifer 163 was virgin and free from *T. foetus* and from April 9 to June 23 she had not been exposed, she must have acquired the

[6] See footnote 2.

infection from the bull on April 9, and therefore this bull must have been infected.

The reason for the lack of data on the method of exposure and length of the incubation period of heifer 161 is as follows: On May 18, when bull 150 served this heifer, it was not known that the bull was infected and consequently the heifer was inoculated, as shown in table 1. Therefore, infection may have been acquired through either coitus or inoculation or by both methods. For 9 months following service on May 18, heifer 161 was considered to be pregnant. However, on March 4, 1938, about a month after expected parturition, a rectal examination was made which led to a tentative diagnosis of pyometra. On March 7, by the use of all known precautions against sepsis, a cannula was placed through the cervical canal and 13½ quarts of pus was aspirated from the uterus. The pus contained leucocytes and trichomonads but was free from bacteria. On March 27, 7½ quarts of foul-smelling pus, which contained bacteria, was aspirated. From February 1938 to March 4, 1939, there were remittent vaginal discharges containing *T. foetus*.

The fetus of cow 158 that aborted on the two hundred and forty-fifth day of gestation weighed 42 pounds and was fully haired. Material from both fetus and placenta was free from trichomonads. Therefore, the abortion was probably not due to trichomoniasis.

RESULTS WITH BULLS

On October 6 and 7 and on November 27, 1936, bull 150 served heifer 157 after the latter had been exposed to trichomonad infection. On September 3 and October 7, 1936, and on January 14 and 21, 1937, he served heifer 160. Apparently during one of these services this bull became infected with *Trichomonas foetus*, although, as stated previously, a positive diagnosis was not made until June 23, 1937. These data show that *T. foetus* was transmitted from a heifer to a bull.

Table 1 shows that after the infection was acquired, bull 150 served each of eight females either as heifers or as cows, one or more times. He served a ninth female, both as a heifer and as a cow. Without exception transmission followed coitus regardless of whether the female in question had or had not been previously infected.

In eight cases following coitus the incubation periods were 12, 11, 14, 13, 11, 11, 6, and 11 days. The average period of incubation was slightly more than 11 days. In three animals infected by intravaginal inoculation the incubation periods were 2, 2, and 4 days.

In comparing the infection of the male bovine host with that of the female it will be noted that with one exception the latter recovered from the disease and exhibited resistance to reinfection. On the other hand, the male has been continuously infected for 20 months, and at present the infection appears to be permanent.

Trichomonad infection had no appreciable effect on the potency of this bull as evidenced by the undelayed conception of reinfected cows 157, 158, 160, and 163.

On October 2, 1936, and February 5, 1937, bull 149 served heifer 158. On October 7, 1936, he served heifer 160, and on February 9, 1937, heifer 157. In every case the service took place after the heifers had been exposed to *Trichomonas foetus*. To March 4, 1939, bull 149 has not become positive for trichomonads.

TABLE 1.—*Results of experimental infection with Trichomonas foetus of 10 bovine animals*

Animal designation	Method of exposure	Dates of transmission	Incubation period	Dates when first and last positive examinations occurred	Period during which examinations were positive for *T. foetus*	Examinations positive			Examinations negative	Total examinations	Bull used		Date of coitus	Conception (+) or nonconception (−)	Interval between heat periods	Date of calving	Approximate time between first coitus and calving	Remarks
						Immediate	After incubation	Total positive			No.	Infected (+) or noninfected (−)						
			Days		*Days*	*No.*	*No.*	*No.*	*No.*	*No.*					*Days*		*Months*	
No. 155: As a heifer											149		Sept. 6, 1936	+		June 21, 1937	9	Control; not infected.
As a cow	Coitus	Sept. 25, 1937	13	Oct. 8, 1937, and Feb. 21, 1938.	136	4	16	16	14	30	150	+	Sept. 25, 1937	−			16½	Bull 150 positive sometime early in 1937. See text.
											150		Mar. 8, 1938	−	164			
											150		Mar. 17, 1938	−	9			
											150		Mar. 24, 1938	−	7			
											150		May 11, 1938	+	48	Feb. 14, 1939		
No. 156: As a heifer											149		Sept. 11, 1936	+		June 11, 1937	9	Control; not infected.
As a cow	Coitus	Sept. 24, 1937	14	Oct. 8, 1937, and Feb. 21, 1938.	136	8	5	8	22	30	150	+	Sept. 24, 1937	−			13½	
											150		Jan. 31, 1938	+	129	Nov. 3, 1938		
No. 157: As a heifer	Inoculation	Sept. 14, 1936; Oct. 7, 1936; Oct. 9, 1936; Oct. 12, 1936	(1)	Oct. 13, 1936, and Jan. 4, 1937.	83	16	20	20	12	32	150	−	Oct. 6, 1936	−				
											150		Oct. 7, 1936	−				
											150		Nov. 27, 1936	−	51			
											149		Feb. 9, 1937	+	74	Nov. 12, 1937	13	
As a cow	Coitus	Mar. 22, 1938; Apr. 4, 1938	11	Apr. 2 and Apr. 22, 1938.	20	1	5	5	8	13	150	+	Mar. 22, 1938	−			9½	
											150		Apr. 4, 1938	+	13	Jan. 2, 1939		
No. 158: As a heifer	Inoculation	Oct. 2, 1936; Oct. 7, 1936	(1)	Oct. 8 and Dec. 30, 1936.	83	31	30	31	2	33	149	−	Oct. 2, 1936	−			13	
											149		Feb. 5, 1937	+	126	Nov. 7, 1937		
As a cow	Coitus	Apr. 20, 1938	6	Apr. 26, 1938	1	1	1	1	0	1	150	+	Apr. 20, 1938	−				Aborted Dec. 30, 1938; not caused by *T. foetus*.
											150		Apr. 29, 1938	+	9			
No. 159 (heifer)	Inoculation	Aug. 22, 1936	2	Aug. 24 and Sept. 14, 1936.	21	6	6	6	0	6								Nonbreeder. Slaughtered Dec. 9, 1936.

No. 160:																		
As a heifer	do	do	2	Aug. 24 and Dec. 30, 1936.	128	12	12	12	32	44	150	−	Sept. 3, 1936	−				
											149	−	Oct. 7, 1936	−	34			
											150	−	Jan. 14, 1937	−	99			13½
											150	−	Jan. 21, 1937	+	7	Oct. 24, 1937		
As a cow	Coitus	Mar. 13, 1938	12	Mar. 25 and Apr. 16, 1938.	22	11	12	12	1	13	150	+	Mar. 13, 1938	−		Dec. 22, 1938	9	
	Coitus	May 18, 1937																
No. 161 (heifer)	Inoculation	May 19, 1937 May 21, 1937 May 24, 1937 May 27, 1937	(1)	June 1, 1937, and Mar. 4, 1939.	641	59	69	69	61	130	150	+	May 18, 1937	+				Pyometra.
No. 162:																		
As a heifer	do	May 28, 1937	4	June 1 and July 9, 1937.	38	15	14	15	1	16	169	−	May 8, 1937	+		Feb. 16, 1938	9	Pregnant when first exposed to infection.
As a cow	Coitus	Apr. 7, 1938	11	Apr. 18 and May 18, 1938.	30	17	21	21	0	21	150	+	Apr. 7, 1938	−				
											150		June 10, 1938	+	64	Mar. 13, 1939	11	
No. 163:																		
As a heifer	do	Apr. 9, 1937	(1)	June 23 and July 1, 1937.	(1)	1	3	3	2	5	150	+	Apr. 9, 1937	−				
											150		July 2, 1937	−	84			12½
											150		July 19, 1937	+	17	Apr. 26, 1938		Diagnosis delayed, see text.
As a cow	do	July 26, 1938	11	Aug. 6 and Aug. 11, 1938.	5	2	2	2	2	4	150	+	July 26, 1938	+		Apr. 30, 1939	9	
No. 174 (heifer)	do	Sept. 13, 1937	11	Sept. 24 and Oct. 28, 1937.	34	8	7	8	5	13	150	+	Sept. 13, 1937	−				
											169	−	Jan. 27, 1938	+	136	Nov. 5, 1938	13½	
Total						192	223	229	162	391								

[1] Not known.

On January 27, 1938, bull 169 served heifer 174 after she had been positive for *Trichomonas foetus*; this bull has likewise shown no evidence of infection. These data appear to indicate that experimental infection of bulls may be difficult.

DISCUSSION

The data on 8 of the 15 infected cases in the female host serve to confirm and amplify those of previous investigators. The infections had one of the following consequences: (1) Failure of early conception and temporary loss of periodicity of oestrum followed by final overcoming of the infection and the restoration to a normal condition, or (2) conception followed by the death of the fetus in utero and the development of pyometra.

The data on the remaining seven cases bring new facts to light, as follows: After infection with *Trichomonas foetus* a heifer failed entirely to breed; however, additional data are needed to determine whether this failure was the result of infection or whether the animal was sexually undeveloped. In a pregnant bovine *T. foetus* established itself in the vagina but was apparently unable to reach the uterus through the cervical seal; as a cow, this animal did not show appreciable resistance to reinfection. Other females that contracted the disease as heifers either before or at the time of coitus showed, as cows, marked resistance to the disease; the infection did not appreciably delay conception.

The data give no indication of a difference in the course of the disease whether transmitted by coitus with an infected bull or by intravaginal inoculation with bacteria-free and virus-free cultures of *Trichomonas foetus*.

Data on the infection in the male bovine host show that after an incubation period of about 11 days a vaginal infection developed in every female served by an infected bull.

The resistance of cows to reinfection after having overcome attacks of the disease as heifers is the most encouraging aspect of the problem. From such resistant cows, calves may be sired by infected bulls without delay of conception or other consequences that usually follow the breeding of heifers and susceptible cows to infected bulls. Further work is needed, however, to determine the duration of the resistance in cows. Infection of heifers and cows is a matter of grave concern, since even in mild cases trichomonad infection lowers the fecundity of the herd. In severe cases, in which pyometra may develop, the fecundity of the female may be destroyed.

The most serious aspect of bovine venereal trichomoniasis is the infection of the bull, for the following reasons: (1) Once acquired, the infection appears to be permanent; (2) transmission by the bull may be expected to occur at every act of coitus, and even semen from an infected bull used for artificial insemination has been shown to result in transmission of the disease[7]; (3) no method of treatment for the elimination of *Trichomonas foetus* in bulls has thus far been developed.

CONTROL OF TRICHOMONIASIS IN HERDS

The following recommendations for the control of trichomoniasis in an infected herd are based on the data obtained in the present investi-

[7] GARLICK, GEORGE G. TRANSMISSION OF BOVINE VENEREAL TRICHOMONIASIS THROUGH ARTIFICIAL INSEMINATION. VET. MED. 34 (1): 42-44. 1939.

gation: (1) Breed infected bulls only to infected cows or to those that have recovered from the infection; (2) breed clean bulls to heifers and to cows known to be free from infection; (3) breed cows with unknown histories artificially with semen from noninfected bulls.

SUMMARY

Fifteen cases of experimentally transmitted trichomoniasis are described in heifers and cows. In 10 of the cases there had not been any previous infection, and in 5 cases there was previous infection. In general, the cases of first infection were severe and those of second infection were mild. One case is described in a bull.

In one heifer transmission of *Trichomonas foetus* during pregnancy resulted in no ill effects. Another infected heifer did not show indications of oestrum. In seven cases infection of the females resulted in failure of early conception and in irregularity or temporary absence of oestrum. The infection was finally overcome and conception and normal gestation followed. In one heifer conception was followed by pyometra. One cow that had a vaginal infection as a heifer showed slight resistance to reinfection. In four cows, reinfection followed reexposure by coitus with an infected bull but without evident ill effects.

By intravaginal inoculation the incubation period was as short as 2 days; by coitus the incubation period averaged about 11 days. The data do not indicate that the method of transmission had any influence on the course of the disease.

Examinations of material from the vaginas of infected females revealed *Trichomonas foetus* in 58 percent of the tests.

Trichomoniasis has persisted for 20 months in a bull infected during coitus with an infected heifer.

Transmission of *Trichomonas foetus* occurred in every female served by an infected bull.

Infection of a bull with *Trichomonas foetus* has had no apparent effect on the animal's potency.

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RELATION OF GAIN IN WEIGHT TO GAIN IN ENERGY CONTENT OF GROWING CHICKS[1]

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INTRODUCTION

The gain in weight, or the quantity of feed required to make a definite amount of gain, is frequently used to compare the values of two or more rations or to judge the efficiency of one feed as compared with that of another. The literature on this subject is voluminous, and need not be reviewed in detail here. The gain in live weight has been used for comparing the efficiency of feeds or rations for growing chicks (*8*),[2] for fattening calves (*5*), lambs (*6*), and other animals (*7*), and for comparing the efficiency of proteins (*2*) or the deficiency of amino acids in proteins. Armsby (*1*) has pointed out that the energy content of equal weights of animal at different periods of fattening may be different, and Fraps (*3*) has shown that the productive energy required per pound of gain in weight is different in different feeding experiments.

Data collected in connection with a study of the utilization of the energy of feeds by chickens [3] have shown that the relation of gain in live weight to feed eaten is not always a reliable measure of the relative energy values of feeds. Some of these data are presented and discussed.

PROCEDURE

The work was done with White Leghorn baby chicks, fed individually. The chicks (2 or 3 days old) were all fed a corn-meal ration for a preliminary period of about 7 days, and then divided into six groups. One group was killed and analyzed for protein and fat. Four groups, having equal average weights, were fed individually in battery brooders on the four rations that were to be compared. The sixth group was used for digestion experiments on the rations to be compared. The chicks were weighed weekly. At the end of the period, usually 21 days, the chicks were killed, the intestinal contents removed, paper pulp or some preservative was added, and an analysis made for protein and fat. The energy content of the chicks was estimated in Calories by the use of the factors 5.66 for fat and 9.35 for protein (*4*).

A corn-meal ration was used as the standard for comparison in experiments 6, 8, and 9. It consisted of corn meal, 56 percent; alfalfa-leaf meal, 6 percent; casein, 12 percent; yeast, 2 percent; wheat gray shorts, 20 percent; calcium carbonate, 1 percent; tricalcium phosphate, 1 percent; salt, 1 percent; and fish oil, 1 percent. For experiments 11, 12, 13, and 17 the standard ration was the same except that 0.2 percent of fortified fish oil was used instead of 1 percent as in the previous ration, and 56.8 percent of corn meal instead of 56 percent.

[1] Received for publication June 12, 1939. Technical contribution No. 529 of the Texas Agricultural Experiment Station.

[2] Italic numbers in parentheses refer to Literature Cited, p. 781.

[3] FRAPS, G. S., and CARLYLE, E. C. UTILIZATION OF ENERGY OF WHEAT PRODUCTS BY CHICKENS. Jour. Nutrition, 1939, 18: In press.

In experiments 6, 8, and 9, 50 percent of corn meal was replaced by 50 percent of patent flour, or low-grade flour, or wheat bran, or wheat brown shorts, for the other rations. In experiments 11 and 12, 50.8 percent of corn meal was replaced by 50.8 percent of starch in one ration, 38 percent of corn meal was replaced by 38 percent of casein in a second ration, and 15 percent of corn meal was replaced by 15 percent of Wesson oil in a third ration. In experiment 13 corn meal was replaced by three different lots of corn bran. In experiment 17, 50 percent of corn meal was replaced by oat hulls or two kinds of kafir.

DATA AND DISCUSSION

Table 1 shows the average gains in live weight of the six chicks in each group, the percentages of protein and fat, the Calories per 100 grams, the gain in energy of the chicks, and the quantities of feed eaten. The data show that the differences in the rations may cause wide differences in the fat content of the chicks. In experiment 6 the chicks fed on the wheat-bran ration contained only 2.96 percent of fat, while those on the corn-meal ration contained 8.81 percent of fat, although the chicks ate considerably more of the wheat-bran ration than of the corn-meal ration. In experiment 8 the fat content was 3.70 percent in the chicks fed the wheat-brown-shorts ration and 8.04 percent for those on the corn-meal ration. Similar differences in the fat content are observed in the other experiments. The highest average fat content (12 percent) was found in experiment 12 from a ration in which 15 percent of corn meal was replaced by 15 percent of Wesson oil. The lowest average fat content (2.02 percent) was found in chicks fed a wheat-bran ration. These differences in fat content mean corresponding differences in energy content. The differences in energy content are not so great as those in fat content, however, since a large portion of the energy comes from the protein and the variations in protein content are both small and in the opposite direction to the variations in fat. That is, the protein slightly increases as the fat decreases. The energy content per 100 gm. is given in table 1, and the differences are appreciable. In experiment 6 the calories range from 196 per 100 gm. for chickens on the corn-meal ration to 146 calories per 100 gm. for those on the wheat-bran ration. The lowest is 144 calories in experiment 17 and the highest is 225 per 100 gm. in experiment 12. Thus, 1 gm. of live weight from chickens fed on one ration may represent much less energy than 1 gm. from chickens fed on another ration.

The grams of feed required to produce 1 gm. of gain and also 1 calorie of gain are given in table 2. The relative values are brought out more clearly when the rations are compared with the corn-meal ration as a standard equal to 100.

The relative quantity of feed required per calories of gain is greater than that required per gram of live weight in all the tests except those with the starch ration and the Wesson-oil ration in experiments 11 and 12. That is to say, the differences in most of the rations are greater than are indicated by the gains in live weight. The differences are especially large with the wheat-bran ration (experiments 6 and 9), the wheat-brown-shorts ration (experiment 8), the oat-hull ration (experiment 17), the corn-bran ration (experiment 13), and the casein ration (experiment 11). The quantity of feed required per gram of

gain in weight and that required per calorie of gain are parallel in most of the experiments, but there are some notable exceptions. Fed on the two Wesson-oil rations, in which 15 percent of corn meal was replaced by 15 percent of Wesson oil (experiments 11 and 12), the chicks required 11 and 13 percent more of the Wesson-oil ration than of the corn-meal ration to make the same gain in live weight, which is contrary to what would reasonably be expected from a ration with a higher feeding value. But for equal calories of gain, the Wesson-oil ration consumed was 95 or 84 percent of the corn-meal ration consumed. The chicks fed the Wesson-oil ration ate less and made less growth than those fed the corn-meal ration, but they stored much greater percentages of fat. The chicks fed the casein ration in experiment 12 required practically the same quantity of feed as those fed the corn-meal ration, in order to make the same gain in live weight, but they contained less fat and required 47 percent more feed to make the same gain in calories.

TABLE 1.—*Average live weight, composition, and gain of energy of chicks and feed eaten*

Experiment No.	Ration	Live weight		Composition		Energy per 100 grams of empty weight	Gain of energy	Feed eaten
		Beginning	Gain	Protein	Fat			
		Grams	*Grams*	*Percent*	*Percent*	*Calories*	*Calories*	*Grams*
6	Corn meal	65.9	125.8	20.09	8.81	196	264.4	275.2
	Patent flour	65.6	122.0	20.14	7.75	186	223.6	271.9
	Low-grade flour	65.9	115.7	20.34	6.15	173	209.1	277.2
	Wheat bran	65.8	111.4	20.84	2.96	146	151.8	356.5
8	Corn meal	60.5	149.2	20.34	8.04	190	289.2	288.6
	Patent flour	59.6	135.1	20.93	7.04	184	255.0	299.8
	Low-grade flour	59.9	141.1	20.79	5.04	165	224.9	289.2
	Wheat brown shorts	60.4	139.5	21.03	3.70	154	200.4	376.1
9	Corn meal	59.4	157.1	21.45	6.66	184	291.3	322.8
	Corn bran	60.2	155.0	23.04	3.13	160	236.6	442.2
	Wheat brown shorts	60.6	148.5	22.94	2.97	158	226.5	362.2
	Wheat bran	60.4	132.6	22.69	2.02	147	178.0	423.3
11	Corn meal	62.5	150.1	20.97	7.38	188	286.1	315.7
	Casein	62.9	94.0	21.62	2.62	147	120.3	220.0
	Starch	63.3	98.0	19.96	9.58	203	217.3	270.1
	Wesson oil	63.0	99.9	20.25	9.87	207	227.0	237.0
12	Corn meal	60.0	130.5	21.04	7.55	190	251.3	280.0
	Casein	60.6	102.2	21.39	2.84	148	129.9	213.1
	Starch	60.1	77.7	19.59	9.31	198	166.5	238.7
	Wesson oil	60.0	91.6	19.97	12.00	225	231.9	217.4
13	Corn meal	53.1	132.1	19.98	8.14	189	258.2	275.4
	Corn bran	52.7	137.0	21.33	3.78	156	207.0	363.8
	do	52.5	141.1	21.39	4.89	167	230.8	346.1
	do	52.9	130.5	21.38	4.16	160	197.6	415.1
17	Corn meal	52.3	124.4	20.59	8.41	195	264.3	275.7
	Oat hulls	52.9	111.7	21.49	2.36	144	154.7	392.9
	Blackhull kafir	53.0	130.2	20.54	8.26	193	269.0	290.0
	"Waxy endosperm" kafir	54.1	121.1	20.72	7.46	187	245.0	275.3

It is evident from the above discussion that gains in live weight or feed required per unit gain in live weight may not be correct measures of the energy values of feeds used in feeding experiments, since the energy contained in the gains may be different with the different groups of animals. The animals may contain different percentages of fat. The quantity of feed required per gram of gain would then not be in the same proportion as the quantity required per calorie of gain. Rations which appear to have the same values when gains in live weight are compared may have different values when gains in energy are compared.

TABLE 2.—*Feed required per gram of gain in live weight or calories*

Experiment No.	Ration	Feed required per gram of gain	Feed required per calorie of gain	Relative quantity required for gain in—	
				Weight	Calories
		Grams	*Grams*	*Grams*	*Grams*
6	Corn meal	2.19	1.04	100	100
	Patent flour	2.23	1.22	102	117
	Low-grade flour	2.40	1.33	110	127
	Wheat bran	3.20	2.35	146	226
8	Corn meal	1.93	1.00	100	100
	Patent flour	2.22	1.18	115	118
	Low-grade flour	2.05	1.29	106	129
	Wheat brown shorts	2.70	1.88	139	188
9	Corn meal	2.06	1.11	100	100
	Corn bran	2.85	1.87	139	169
	Wheat brown shorts	2.44	1.60	119	144
	Wheat bran	3.19	2.38	155	215
11	Corn meal	2.11	1.10	100	100
	Casein	2.34	1.83	111	166
	Starch	2.76	1.24	131	113
	Wesson oil	2.37	1.04	113	95
12	Corn meal	2.15	1.11	100	100
	Casein	2.09	1.64	97	147
	Starch	3.07	1.43	143	129
	Wesson oil	2.37	.94	111	84
13	Corn meal	2.09	1.07	100	100
	Corn bran	2.66	1.76	127	165
	....do	2.45	1.50	118	141
	....do	3.18	2.10	153	197
17	Corn meal	2.22	1.04	100	100
	Oat hulls	3.52	2.54	159	244
	Blackhull kafir	2.23	1.08	100	103
	"Waxy endosperm" kafir	2.27	1.12	102	108

The comparison of gains in live weight is convenient and practical, and no better method may be available for some purposes, especially for practical comparisons in feeding experiments. The possible inaccuracies here pointed out, however, should not be disregarded. In scientific work, the differences in energy content should be taken into consideration. Feed per calorie of gain is also not an exact method of comparison, since the feed used for maintenance is not considered. The maintenance requirements are allowed for when the productive energy is estimated (*4*, *5*).

SUMMARY

The fat and energy content of chicks fed rations in which the feed tested replaced corn meal, were determined in six series of experiments. The fat content of the chicks ranged from a minimum of 2.02 percent to a maximum of 12.02 percent and the energy content from 144 to 225 calories per 100 gm. The energy content per gram of live weight was different for the different rations. The relative quantity of feed required per gram of gain in live weight was different from the relative quantity required per calorie of gain. In one experiment the chicks on a casein ration required practically the same quantity of feed as those on a corn-meal ration per unit of gain in live weight, but they required 47 percent more per calorie of gain. Chicks fed a ration in which 15 percent of Wesson oil replaced 15 percent of corn meal required in two experiments 11 or 13 percent more feed to produce the same gain in live weight as the corn-meal ration, although the Wesson-oil ration should have a higher productive energy. They required 5 or 16 percent less of the Wesson-oil ration to produce the same calories of gain, which is in accord with the higher productive

energy. Feed required per unit of gain in live weight is not a safe standard for comparing the feeding values of rations, though it may be a good practical one, especially for animals sold on a weight basis. In scientific work, the fact that equal gains in live weight do not necessarily mean equal gains in energy should not be overlooked.

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